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Integrating Functional Hydrogel Microstructures into Micropatterned Hepatocellular Co-Cultures

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Abstract

This paper describes a microfabrication-derived approach for defining interactions between distinct groups of cells and integrating biosensors with cellular micropatterns. In this approach, photoresist lithography was employed to micropattern cells adhesive ligand (collagen (I)) on silane-modified glass substrates. Poly(ethylene glycol) (PEG) photolithography was then used to fabricate hydrogel microstructures in registration with existing collagen (I) domains. A glass substrate modified in this manner had three types of micropatterned regions: cell-adhesive collagen (I) domains, moderately adhesive silanized glass regions and non-adhesive PEG hydrogel regions. Incubation of this substrate with primary rat hepatocytes or HepG2 cells resulted in attachment of hepatic cells on collagen (I) domains with no adhesion observed on silane-modified glass regions or hydrogel domains. 3T3 fibroblasts added onto the same surface attached on the glass regions around the hepatocytes, completing the co-culture. Significantly, PEG hydrogel microstructures remained free of cells and were used to “fence” hepatocytes from fibroblasts, thus limiting communication between the cell types. We also demonstrated that entrapment of enzyme molecules inside hydrogel microstructures did not compromise non-fouling properties of PEG. Building on this result, horse radish peroxidase (HRP)-containing hydrogel microstructures were integrated into micropatterned co-cultures and were used to detect hydrogen peroxide in the culture medium. The surface micropatterning approach described here may be used in the future to simultaneously define and detect endocrine signaling between two distinct cell types.

1. Introduction

Surface micropatterning approaches have been used extensively for defining cellular interactions and creating miniature *in vitro* tissue models.¹⁻⁴ Micropatterned surfaces created by a variety of methods, including microarraying, microcontact printing, photoresist lithography and microfluidics, typically contain “cell-adhesive” domains interspersed with “non-adhesive” regions. Depending on the selection of surface chemistry and cell types these “binary” surfaces may be used to create micropatterned mono- and co-cultures.^{3, 5-7} However, increasing the number of cell types assembled on the same surface and/or manipulating the interactions between the different cell types requires enhancing biointerface complexity beyond the “binary” micropattern. Several groups have employed electrochemistry to modulate

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surface properties in a sequential manner, thus allowing to define local surface properties and to guide attachment of the desired cell types.⁸⁻¹² Hui and Bhatia have recently described the use of micromachined silicon substrates for modulating interactions between cell types of the co-culture in both spatial and temporal fashion.¹³ The same group has described another surface modification method employing a series of photolithography, protein adsorption and PEG silane modification steps to create three component substrates containing cell non-adhesive, moderately adhesive and strongly adhesive regions.¹⁴ These surfaces were then employed to define interactions of hepatocytes and fibroblasts within micropatterned co-cultures.

Our study explored the possibility of merging photoresist lithography and PEG hydrogel photopatterning in order to register protein and biomaterial micropatterns, thus enabling more intricate control of cell attachment. In contrast to bottom-up PEG-silane or PEG-thiol surface modification approaches, PEG hydrogel photopatterning (“photolithography”) is a top-down process that resembles negative tone resist patterning and therefore allows for seamless integration with other steps of the microfabrication process.¹⁵⁻¹⁷ As shown by us and others,¹⁶⁻²⁰ hydrogel micropatterns have excellent non-fouling properties and can be used to effectively control cell attachment. However, unlike nanometer-scale self-assembled PEG films that also exhibit excellent resistance to protein or cell adhesion, PEG hydrogel elements have micrometer-scale thickness and have been used extensively as matrices for entrapment of sensing molecules such as enzymes.^{15, 21-24} While PEG hydrogel microstructures have been employed for cellular micropatterning and biosensing in separate studies, the combination of these two applications in the same hydrogel micropattern has not been demonstrated to the best of our knowledge.

The present paper describes a surface modification process that employed hydrogel micropatterning in concert with photoresist lithography in order to control interactions between two cell types and integrate biosensors at the site of the cells. PEG hydrogel microstructures were photopatterned in registration with collagen regions, creating a complex biointerface with: 1) hepatocyte-adhesive collagen (I) regions, 2) fibroblast-adhesive acrylated silane regions and non-adhesive PEG hydrogel domains. Exposing a micropatterned glass substrate to hepatocyte cell suspension resulted in selective attachment of these cells on collagen (I) regions of the surface. Subsequent seeding of fibroblasts resulted in adhesion of these cells on glass substrates around the hepatocytes, thus completing the co-culture. PEG hydrogel microstructures remained free of cells and could be used to segregate one cell type from the other. In addition, the same hydrogel microstructures could serve as biosensors of the cellular environment. This was demonstrated by integrating horseradish peroxidase (HRP)-containing hydrogel microstructures with micropatterned co-cultures and detecting exogenous hydrogen peroxide in the culture medium. The surface micropatterning approach described here allowed intimate integration of cells with biosensing elements and may be used in the future to simultaneously define and detect endocrine interactions between two cell types.

2. Materials and methods

2.1. Chemicals and materials

Glass slides (75×25 mm) were obtained from VWR international. 3-acryloxypropyl trichlorosilane was purchased from Gelest, Inc. Sulfuric acid, hydrogen peroxide, ethanol, toluene, collagen from rat tail (type I), epidermal growth factor, bovine serum albumin, horseradish peroxidase (HRP), poly(ethylene glycol) diacrylate (PEG-DA) (MW 575), 2,2'-dimethoxy-2-phenylacetophenone (DMPA) were obtained from Sigma-Aldrich. Dimethyl sulfoxide (DMSO) was from Pierce (Rockford, IL). Phosphate-buffered saline (PBS) 10× was purchased from Cambrex. Dulbecco's modified Eagles' medium (DMEM), minimal essential medium (MEM), sodium pyruvate, non-essential amino acids, L-glutamine, fetal bovine serum (FBS), Amplex® Red and collagen (I) - FITC reagent were purchased from Invitrogen Life

Technologies. Glucagon and insulin were obtained from Eli-Lilly, and hydrocortisone was obtained from Pfizer. FluoroLink™ Cy3 reactive dye 5-pack was purchased from Amersham Bioscience. CellTracker™ Green CMFDA and Red CMTPX probes for long-term tracing of living cells were purchased from Molecular Probes.

2.2. Silane treatment of glass substrates

The glass slides were cleaned by immersion in piranha solution consisting of a 1:1 ratio of 95% v/v sulfuric acid and 35% w/v of hydrogen peroxide for 10 minutes. Glass slides were thoroughly rinsed with deionized (DI) water, and then dried under nitrogen. For silane modification, the glass slides were treated in an oxygen plasma chamber (YES-R3, San Jose, CA) at 300 W for 5 minutes and then placed in a solution containing 3-acryloxypropyl trichlorosilane diluted in anhydrous toluene (20 μ l/40 ml) for 10 minutes. The reaction was performed in a glove box filled with nitrogen to eliminate atmospheric moisture. The slides were rinsed with fresh toluene, dried under nitrogen, and cured at 100°C for 2 hours. The silane-modified glass slides were stored in a desiccator before use.

2.3. Micropatterning proteins using photoresist lithography

Silane-modified glass slides were micropatterned to fabricate fiduciary marks. AZ 5214-E positive photoresist was spin-coated on the silane-modified glass substrate at 800 rpm for 10 seconds followed by 4,000 rpm for 30 seconds. The coated slide was soft-baked on a hot plate at 100 °C for 85 seconds. The photoresist layer was exposed to UV light (10 mW/cm²) for 35 seconds using a Canon PLA-501F Mask Aligner. Exposed photoresist was then developed for 5 minutes in AZ 300 MIF developer solution, briefly washed with DI water to remove residual developing solution, and then dried under nitrogen. A 500 Å aluminum (Al) layer was deposited onto glass slides with patterned photoresist using an E-beam Evaporator (Fremont, CA). The glass slides were then placed into acetone and sonicated for 20 minutes to lift off Al layer atop the photoresist, and thereby creating Al alignment marks.

In order to create collagen (I) micropatterns, silanized glass slides containing metal alignment marks were patterned with photoresist using the recipe described above. Registration of the photoresist layer with fiduciary marks was ensured with either a mask aligner or an upright microscope. A glass slide containing photoresist pattern was then incubated in solution of collagen (I) (0.1 mg/ml in 1× PBS) for 30 minutes at room temperature. A slide was then briefly washed with DI water, sonicated in acetone for 10 min to remove the photoresist and dried under nitrogen. The glass slides containing collagen patterns were kept at 4°C prior to use.

2.4. Characterization of protein micropatterns

The quality of protein micropatterns was first assessed by fluorescence microscopy. For the visualization of the patterns, collagen (I) was labeled with Cy3 using FluoroLink™ Cy3 reactive dye 5-pack according to the manufacturer's instruction. The labeled protein pattern was observed under LSM 5 Pascal confocal microscope (Carl Zeiss Inc) using excitation/emission (ex/em) wavelengths of 552/565 nm. The topology of protein micropatterns was analyzed using imaging ellipsometry. For these experiments, collagen (I) patterns were created on silane-modified silicon wafers using photoresist lithography protocols detailed above. The measurements were taken using an iElli2000 imaging null ellipsometer (Gottingen, Germany) with a 20 mW Nd:YAG frequency doubled laser operating at 1% power. Spatial maps of the ellipsometric parameter, delta, were acquired using a 10× objective giving a field of view of 645×430 μ m and a lateral resolution of ~2 μ m. Delta maps were acquired using a series of 70 images collected over 4 degrees of polarizer rotation. The null conditions of each pixel were then determined from this series of images and used to calculate delta values. Topographical maps of thickness were generated from these delta values using an optical model that assumed isotropic parallel slabs and a refractive index of 1.47 for both the acrylated silane and collagen.

Root Mean Square (RMS) measurements were made on four 25×25 pixel separate regions on collagen and silane to characterize the roughness of the organic layer.

2.5. Fabricating PEG hydrogel microstructures in registration with collagen micropatterns

Photolithographic patterning of PEG hydrogel was described by us earlier.¹⁶ Briefly, a precursor solution was prepared by mixing PEG-DA (MW 575) with 1% (w/v) photoinitiator (DMPA). This solution was spin-coated at 600 rpm for 5 seconds onto glass surface containing acrylated silane layer. A photomask was placed on top of the liquid layer of PEG-DA precursor solution and aligned to fiduciary marks (indicators of collagen domains) using an upright microscope or a mask aligner. Then the sample was exposed through a chrome/soda lime photomask to UV for 1.5 seconds at 60 mJ/cm² using OmniCure™ Series 1000 light source or 10 sec using 10 mJ/cm² Cannon PLA-501F Mask Aligner. The regions of PEG-DA exposed to UV light underwent free-radical polymerization and became cross-linked, while unexposed regions were dissolved in DI water after 5 min of development. High-resolution images of PEG microstructures were obtained using a Philips XL 30 Scanning Electron Microscope at 10 kV beam voltage and a tilt angle of 40°. Prior to SEM imaging, the substrates were sputter-coated with ~10 nm of Au-Pd. The same sample preparation was used to image hydrogel patterns with and without cells.

2.6. Creating micropatterned cell cultures

Primary rat hepatocytes or human hepatoma (HepG2) cells were used in our experiments. Rat hepatocytes were isolated and purified according to established protocols²⁵. Hepatocytes were maintained at 37 °C in a humidified 5% CO₂ atmosphere in DMEM supplemented with 10% FBS, 200 U/ml penicillin, 200 µ/ml streptomycin, 7.5 µ/ml hydrocortisone, 20 ng/ml epidermal growth factor, 14 ng/ml glucagon, and 0.5 U/ml insulin. HepG2 cells were maintained in MEM supplemented with 10% FBS, 200 units/ml penicillin, 200 µ/ml streptomycin, 1 mM sodium pyruvate, and 1 mM nonessential amino acids at 37 °C in a humidified 5% CO₂ atmosphere. Murine 3T3 fibroblasts were maintained in DMEM supplemented with 10% FBS, 200 U/ml penicillin, and 200 µ/ml streptomycin at 37 °C in a humidified 5% CO₂ atmosphere.

For the cell seeding experiments, glass pieces were placed into wells of a conventional 6-well plate. Glass slides were sterilized with 70% ethanol, and washed twice with 1× PBS. Cellular micropatterning process was carried out by exposing collagen/PEG hydrogel patterned slides to 3 ml of hepatocyte suspension in culture medium at a concentration of 1×10⁶ cells/ml. After one hour of incubation at 37 °C, the medium containing unattached cells was removed and surfaces were washed twice with 1× PBS. Cell patterns formed on the glass slide were imaged by brightfield microscope (Carl Zeiss Inc.). To introduce the second cell type, glass slides containing surface-bound hepatocytes were exposed to 3 ml of fibroblast cell suspension at a concentration of 5×10⁵ cells/ml. After 15 minutes incubation at 37 °C, the fibroblast culture medium was removed and replaced with hepatocyte culture medium. Cell patterns formed on a glass slide were imaged by brightfield microscope and high-resolution images of individual cell clusters were obtained using Philips XL 30 Scanning Electron Microscope (SEM). For SEM imaging, the cells were fixed with 4% paraformaldehyde solution and sputter-coated with ~10 nm of Au-Pd.

In order to demonstrate spatial segregation of two cell types, hepatocytes and fibroblasts were labeled with cell tracker dyes prior to seeding. HepG2 cells and 3T3 fibroblasts were labeled with Cell Tracker Green CMFDA (ex/em 492/517nm) and Cell Tracker Red CMTPX (ex/em 577/602nm) respectively according to manufacturer instructions. Two color fluorescence images of the micropatterned co-cultures were obtained using LSM 5 Pascal confocal microscope (Carl Zeiss Inc).

2.7. Detecting model metabolites using enzyme-carrying PEG hydrogel microstructures

HRP stock solution was prepared by dissolving in 1× PBS to make the final concentration at 10 mg/ml. Then HRP stock solution (10%, v/v) was mixed with PEG-DA prepolymer solution (90%, v/v). This HRP-containing precursor solution was micropatterned on the glass substrate using spin-coating, UV exposure and water development process described above. Amplex Red reagent was used as a fluorescence probe to detect HRP-catalyzed oxidation of hydrogen peroxide (H_2O_2). Amplex Red reagent was dissolved in DMSO to final concentration of 5 mM and was stored in a desiccator at -20 °C. For detection of H_2O_2 , Amplex Red solution was added into cell culture medium to create a concentration of 50 μ M. Finally, to demonstrate enzymatic activity of HRP molecules entrapped in PEG hydrogel elements H_2O_2 at a concentration of 10 μ M was added into the cell culture medium. The change in fluorescence of the hydrogel structures was imaged with LSM 5 Pascal confocal microscope using ex/em filters of 563/587 nm.

3. Results and discussion

In this study, PEG hydrogel microfabrication and photoresist lithography were used in concert to precisely position hydrogel structures within micropatterned co-cultures of hepatocytes and fibroblasts. This process consisted of a series of surface micropatterning steps performed in registration followed by sequential seeding of hepatocytes and fibroblasts, as shown in Figure 1. Placing non-fouling PEG hydrogel elements into co-cultures could be used in the future to control interactions between the two cell populations. Incorporation of functional enzymes into PEG microstructures was also demonstrated, underscoring future applications of this fabrication method for sensing cellular microenvironment.

3.1. Creating protein micropatterns with photoresist lithography

Photoresist lithography is a proven method for creating micropatterned biological interfaces and has been used previously for patterning proteins and cells.^{5, 26} Because photolithography originated in semiconductor industry, it was particularly suited for integration into a multi-step surface modification process described here. Prior to photoresist patterning, glass slides were modified with acryloxypropyl trichlorosilane - an adhesion promoting layer used for covalent coupling of PEG hydrogel microstructures to glass during a later microfabrication step. In addition to promoting hydrogel adhesion, acrylated silane was noted by us previously to make glass substrates permissive to attachment of fibroblasts but resistive to adhesion of hepatocytes.^{27, 28} Fibroblasts are most common cells of connective tissue whose main role is to synthesize the extracellular matrix in tissue remodeling and repair. Hence, these cells are expected to produce a number of endogenous proteins and modify local microenvironment rapidly, attaching on silane-functionalized glass substrata. In contrast, hepatocytes are parenchymal cells that do not actively secrete ECM proteins and therefore require substrates pre-coated with ECM components to promote attachment. Therefore, the cell types could be sequentially seeded onto silane-modified glass surfaces to assemble hepatocyte-fibroblast co-cultures. These experiments are described in a later section of the paper.

Photoresist layer was patterned on a glass slide in registration with fiduciary marks to create a stencil for adsorption of collagen (I) as shown in Figure 2A. Fiduciary marks identified regions of protein adsorption and were used for alignment during a subsequent hydrogel micropatterning step. Figure 2B shows micropatterns of Cy3-labeled collagen (I) on the glass surface after photoresist lift-off. Importantly, we have previously investigated the effects of acetone based photoresist lift-off on attachment and albumin synthesis of hepatic cells residing on collagen (I) micropatterns.²⁷ No detriment in cell adhesion and function due to lift-off process was observed in our previous study as well as here. Therefore, there is evidence pointing to physically adsorbed ECM protein molecules remaining on the surface and being at

least at least partially functional after 5-10 min acetone treatment required for the photoresist lift-off.

The topology of collagen micropatterns was characterized using imaging ellipsometry. Imaging ellipsometry is a non-destructive and label-free method which can be used to characterize large surface area with molecular-scale resolution.²⁹⁻³⁴ A representative image from ellipsometric analysis of collagen (I) micropattern adsorbed onto silane-treated silicon substrate is shown in Figure 2C. The layer of acrylated silane was uniform across the sample with a calculated thickness of 1.9 ± 0.2 nm; the value consistent with that of a well-packed monolayer of acrylated silane. The collagen films were smooth and uniform with thickness of 4.3 ± 0.3 nm (2.4 nm above the silane layer) with reasonably sharp (<10 μ m) edges as shown by height profile in Figure 2C. This thickness of collagen closely relates to our previously reported measurement of 3.0 ± 1.0 nm characterized using atomic force microscopy²⁷ and was consistent with previous reports of organization of monomeric collagen (I) layers.³⁵

3.2. Fabricating PEG hydrogel microstructures in registration with collagen micropatterns

PEG-DA has been photopatterned previously to create surfaces for controlling cell attachment with single cell resolution. While soft lithography-based approaches for forming PEG hydrogel microstructures have been reported,²⁰ the top-down approach^{15, 16}, whereby photosensitive PEG prepolymer is spun onto a substrate and exposed to light through a photomask, resembles traditional resist lithography and is amenable to alignment and integration with existing microfabricated layers. Figure 3(A,B) shows representative hydrogel micropatterns comprised of 200 μ m diameter pads interconnected with line width of 40 μ m or PEG rings with 40 μ m width and 200 μ m inner diameter. These SEM images demonstrate the possibility of densely populating large surface area with hydrogel elements and also underscore three-dimensionality of the hydrogel structures with thickness of ~ 40 μ m (see inset of Figure 3A).

In contrast to hydrogel micropatterning results reported by us previously,¹⁵⁻¹⁷ the present study sought to fabricate PEG microstructures in registration with existing protein patterns. The alignment of collagen (I) and hydrogel micropatterns is demonstrated in Figure 3(C,D). The fluorescently labeled proteins were used in these images to highlight the fidelity of registration; however, in routine experiments, non-fluorescent collagen patterns were aligned with hydrogel elements using a photomask and fiduciary marks patterned on the glass substrate. The micropatterning results presented in Figure 3(C,D) are significant as they demonstrate a simple two-mask microfabrication process that allows micropatterning of two biomaterials in registration with each other.

3.3. Micropatterning co-cultures of hepatocytes and fibroblasts

The two mask microfabrication process described diagrammatically in Figure 1 and demonstrated in Figure 3 (A-D) resulted in micropatterned surfaces with repeating domains of cell-adhesive collagen (I), moderately adhesive silanized glass and non-adhesive PEG hydrogel. Hepatocytes have been reported to preferentially attach on adhesive ligands such as collagen (I).^{5, 36} As expected, incubation of a micropatterned substrate with hepatocytes (see Figure 3C) resulted in selective adhesion of these cells on collagen (I) domains with limited cell attachment on silane- or hydrogel-coated glass regions. Figure 3E highlights selective attachment of hepatocytes on collagen islands next PEG hydrogel elements. Importantly, this selective attachment occurred on a large scale as shown in Figure 3F.

The complexity of a cell culture system seen in Figure 3(E,F) could be further increased by adding a second cell type onto the same surface, thus creating micropatterned co-cultures. This particular co-culture assembly process first reported by Bhatia et al.⁵ relies on differences in “adhesiveness” of liver parenchymal cells (hepatocytes) and non-parenchymal cells; the former

require ECM coatings such as collagen (I) while the latter can attach on less hospitable substrates such as BSA- or silane-coated glass. Sequential seeding of hepatocytes followed by the non-parenchymal cells could then be used to assemble micropatterned co-cultures.^{5, 27, 28} Analogous strategy was employed in the present study. Figure 4(A,B) shows higher magnification images of HepG2 cells adherent on collagen (I) domains in registration with PEG hydrogel rings and pads. Incubation of these cellular micropatterns with 3T3 fibroblasts led to attachment of these cells onto silane-modified glass regions around the hepatocyte clusters. Limited attachment of cells of either type was observed on PEG hydrogel structures (Figure 4(C,D)). Fluorescent labeling of hepatocytes (green) and fibroblasts (red) was employed to verify spatial sequestration of the cell types after co-culture assembly. As shown Figure 4(E,F) the two cell populations remained distinct after sequential seeding steps and minimal intermingling of cell types was observed after 5 days in culture (results not shown). Importantly, PEG hydrogel microstructures were free of cells throughout seeding steps and after one week of cultivation (data not shown). Figure 4(D,F), demonstrating distinct groups of hepatocytes (green) and fibroblasts (red) separated from each other by a PEG hydrogel “fence”, points to a potential application of this approach for controlling both physical and endocrine interactions between two distinct cell types.

The nonfouling properties of the hydrogel microstructures do not change appreciably over time in culture. However, rapidly proliferating cells such as 3T3 fibroblasts may “spill over” from the hydrogel containment to form physical contacts with another group of cells. In the present study, hepatocyte-fibroblast co-cultures were maintained for 4 to 5 days without compromising fidelity of the cell micropatterns. In the future, rapidly proliferating cells such as fibroblasts can be growth arrested allowing to extend the life-time of the cell culture to several weeks. Controlling dimensions of the hydrogel microstructures sequestering one cell population from another may be used to both ensure cell containment and to modulate endocrine communication between the cell types.

3.4. Using the same PEG microstructures for cell micropatterning and biosensing

In addition to control of cellular interactions, PEG hydrogel structures have been employed as matrices for entrapment of biosensing elements such as enzymes.^{15, 21, 23, 37} Therefore, we were interested in exploring the possibility of integrating enzyme-containing PEG microstructures with cellular micropatterns. For a proof-of-concept experiment, we chose to detect HRP-catalyzed oxidation of hydrogen peroxide (H_2O_2) with Amplex Red serving as a fluorescence reporter of this reaction. This is a well-established bioassay where Amplex Red, a non-fluorescent compound, is oxidized to a fluorescent product resorufin in the process of HRP-catalyzed breakdown of H_2O_2 .³⁸ Beyond proof-of-concept demonstration, H_2O_2 detection was chosen because of importance of this reactive oxygen species (ROS) in the immuno-inflammatory processes. In order to fabricate biosensing hydrogel microstructures, HRP molecules were mixed into PEG-based prepolymer solution and photopatterned in registration with existing collagen (I) microdomains using spin-coating, alignment and UV exposure steps described above. One question was how entrapment of enzyme molecules will affect fouling properties of PEG hydrogel microstructures? As shown in a representative SEM image in Figure 5A, HRP-containing PEG structures remained resistant to cell-attachment during the seeding of both hepatocytes and fibroblasts. Therefore, incorporation of enzyme did not affect non-fouling properties of PEG structures. Importantly, entrapped HRP-molecules retained enzymatic activity. Figure 5(B,C) shows merged fluorescence/brightfield images of biosensing PEG structures acquired before and after addition of 10 μM H_2O_2 into cell culture media. Amplex Red was present in the cultured media prior to introduction of H_2O_2 . This fact likely accounts for background fluorescence observed in Figure 5B. However, comparison of fluorescence/brightfield images in Figure 5B (no H_2O_2) and Figure 5C (10 μM H_2O_2) clearly demonstrates that HRP-containing PEG microstructures were responsive to exogenous

H₂O₂. Upon addition of analyte the fluorescence signal observed in the hydrogel structures increased rapidly and reaching 40% of intensity after 10 min.

We have examined indirectly how long the encapsulated enzymes retain in the PEG hydrogel using FITC-labeled dextran of similar molecular weight (data not shown). The fluorescence intensity decreased gradually over the culture period, but the intensity at day 4 was measured to 80% of the initial intensity. Given the possibility of fluorescence signal decrease due to photobleaching, the majority of enzyme molecules are likely to be retained in PEG hydrogel mesh after 4 days in culture. In the future, enzyme molecules will be acrylated and covalently cross-linked into hydrogel matrix to further ensure retention of the biosensor function over time.

While the results presented in Figure 5 stop short of detecting endogenous extracellular metabolites, they point to the possibility of placing functional metabolite biosensors at the site of a small, yet well defined and heterogeneous cell population. A microsystem combining cellular micropatterns and miniature biosensors may be employed in the future for detecting endocrine communication between distinct cell populations. Furthermore, enzyme-based biosensors integrated with two distinct cell populations and reporting on the appearance of small analytes may be combined with immunoassays³⁹ and/or laser-mediated retrieval-PCR approaches²⁸ to evaluate changes in cell phenotype in response to an analyte exchange.

4. Conclusions

In this study, we described a surface micropatterning approach whereby photoresist lithography and PEG hydrogel patterning were used to register protein micropatterns and hydrogel microstructures on a glass substrate. This process allowed to integrate non-fouling PEG structures into micropatterned co-cultures of hepatocytes and non-parenchymal cells, thus offering a method for precisely controlling physical and endocrine interactions between two distinct cell types. In addition, we demonstrated the possibility of entrapping functional enzymes into PEG hydrogel microstructures and placing biosensing hydrogel elements into micropatterned co-cultures. Therefore, micropatterning strategy described here may serve a dual role for defining and detecting communication between small groups of cells and may provide a new tool for the investigation of reciprocal cellular interactions.

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REFERENCES

1. El-Ali J, Sorger PK, Jensen KF. *Nature* 2006;442:403–411. [PubMed: 16871208]
2. Park TH, Shuler ML. *Biotech. Prog* 2003;19:243–253.
3. Singhvi R, Kumar A, Lopez GP, Stephanopoulos GN, Wang DIC, Whitesides GM, Ingber DE. *Science* 1994;264:696–698. [PubMed: 8171320]
4. Whitesides GM, Ostuni E, Takayama S, Jiang X, Ingber DE. *Annu. Rev. Biomed. Eng* 2001;3:335–373. [PubMed: 11447067]
5. Bhatia SN, Yarmush ML, Toner M. *J. Biomed. Mater. Res* 1997;34:189–199. [PubMed: 9029299]
6. Bhatia SN, Balis UJ, Yarmush ML, Toner M. *FASEB J* 1999;13:1883–1900. [PubMed: 10544172]
7. Khademhosseini A, Langer R, Borenstein J, Vacanti JP. *Proc. Nat. Acad. Sci. USA* 2006;103:2480–2487. [PubMed: 16477028]
8. Jiang X, Ferrigno R, Mrksich M, Whitesides GM. *J. Am. Chem. Soc* 2003;125:2366–2367. [PubMed: 12603104]

9. Li Y, Yuan B, Ji H, Han D, Chen SQ, Tian F, Jiang XY. *Angew. Chem. Int. Ed* 2007;46:1094–1096.
10. Yeo WS, Mrksich M. *Langmuir* 2006;22:10816–10820. [PubMed: 17129065]
11. Yousaf MN, Houseman BT, Mrksich M. *Proc. Natl. Acad. Sci. USA* 2001;98:5992–5996. [PubMed: 11353818]
12. Shah SS, Lee JY, Verkhoturov S, Tuleuova N, Schweikert EA, Revzin A. *Langmuir* 2008;24:6837–6844. [PubMed: 18512875]
13. Hui EE, Bhatia SK. *Proc Nat. Acad. Sci. USA* 2007;104:5722–5726. [PubMed: 17389399]
14. Hui EE, Bhatia SN. *Langmuir* 2007;23:4103–4107. [PubMed: 17243746]
15. Revzin A, Russell RJ, Yadavalli VK, Koh W-G, Deister C, Hile DD, Mellott MB, Pishko MV. *Langmuir* 2001;17:5440–5447. [PubMed: 12448421]
16. Revzin A, Tompkins RG, Toner M. *Langmuir* 2003;19:9855–9862.
17. Revzin A, Sekine K, Sin A, Tompkins RG, Toner M. *Lab Chip* 2005;5:30–37. [PubMed: 15616737]
18. Revzin A, Rajagopalan P, Tilles AW, Berthiaume F, Yarmush ML, Toner M. *Langmuir* 2004;20:2999–3005. [PubMed: 15875819]
19. Hahn MS, Taite LJ, Moon JJ, Rowland MC, Ruffino KA, West JL. *Biomaterials* 2006;27:2519–2524. [PubMed: 16375965]
20. Suh KY, Seong J, Khademhosseini A, Laibinis PE, Langer R. *Biomaterials* 2004;25:557–563. [PubMed: 14585705]
21. Russell RJ, Pishko MV, Simonian AL, Wild JR. *Anal. Chem* 1999;71:4909–4912. [PubMed: 10565282]
22. Seong GH, Heo J, Crooks RM. *Anal. Chem* 2003;75:3161–3167. [PubMed: 12964765]
23. Allcock HR, Phelps MVB, Barrett EW, Pishko MV, Koh WG. *Chem. Mater* 2006;18:609–613.
24. Zguris J, Pishko MV. *Sens. Lett* 2005;3:206–210.
25. Dunn JCY, Yarmush ML, Koebe HG, Tompkins RG. *FASEB J* 1989;3:174–179. [PubMed: 2914628]
26. Healy KE, Lom B, Hockberger PE. *Biotech. Bioeng* 1994;43:792–800.
27. Lee JY, Shah SS, Zimmer C, Liu GY, Revzin A. *Langmuir* 2008;24:2232–2239. [PubMed: 18198912]
28. Lee JY, Jones CN, Zern MA, Revzin A. *Anal. Chem* 2006;78:8305–8312. [PubMed: 17165820]
29. Bae YM, Oh BK, Lee W, Lee WH, Choi JW. *Biosens. Bioelectron* 2004;20:895–902. [PubMed: 15522607]
30. Bae YM, Oh BK, Lee W, Lee WH, Choi JW. *Anal. Chem* 2004;76:1799–1803. [PubMed: 15018586]
31. Howland MC, Sapuri-Butti AR, Dixit SS, Dattelbaum AM, Shreve AP, Parikh AN. *J. Am. Chem. Soc* 2005;127:6752–6765. [PubMed: 15869298]
32. Schmaljohann D, Nitschke M, Schulze R, Eing A, Werner C, Eichhorn YJ. *Langmuir* 2005;21:2317–2322. [PubMed: 15752021]
33. Wang ZH, Jin G. *Anal. Chem* 2003;75:6119–6123. [PubMed: 14615990]
34. Zhou Y, Andersson O, Lindberg P, Liedberg B. *Microchimica Acta* 2004;147:21–30.
35. Elliott JT, Tona A, Woodward JT, Jones PL, Plant AL. *Langmuir* 2003;19:1506–1514.
36. Caron JM. *Mol. Cell. Biol* 1990;10:1239–1243. [PubMed: 2406565]
37. Lee WJ, Choi DK, Lee Y, Kim DN, Park JW, Koh WG. *Sens. Actuator BChem* 2008;129:841–849.
38. Zhou M, Diwu Z, Panchuk-Voloshina N, Haugland RP. *Anal. Biochem* 1997;253:162–168. [PubMed: 9367498]
39. Jones CN, Lee JY, Stybayeva GS, Zern MA, Revzin A. *Anal. Chem* 2008;80:6351–6357. [PubMed: 18642875]

Figure 1.

Schematic diagram describing surface microfabrication procedure (step 1-5) and cellular assembly (step 6, 7): *Step 1.* Photoresist is lithographically patterned on silanized glass substrates in registration with metal fiduciary marks (not shown). Photoresist serves as stencil during protein adsorption and alignment marks designate regions containing protein. *Step 2.* Collagen (I) is physisorbed onto a glass slide containing photoresist pattern. *Step 3.* Photoresist is removed by sonication in acetone (lift-off) leaving behind micropatterned collagen (I) domains. *Step 4-5.* PEG-DA prepolymer solution was spin-coated, aligned to fiduciary marks on the surface and exposed to UV through photomask. *Step 6-7.* Hepatocytes, seeded onto the surface first, attach selectively to collagen regions, leaving glass and PEG regions free of cells. Fibroblasts were seeded in a second, adhering on glass domains and completing a micropatterned co-culture.

Figure 2.

Using photoresist lithography to pattern collagen (I). (A) A photoresist pattern aligned to metal fiduciary marks (black dots). The size of inner circle was 200 μm in diameter and the width of ring was 50 μm. (B) Collagen micropatterns created by incubating patterned photoresist with protein solution followed by lift-off. (C) A topograph of collagen pattern obtained with imaging ellipsometry. Height profile shows protein layer to be ~2.4 nm in thickness, suggesting that collagen (I) present on the surface was in monomeric state.

Figure 3.

Registration of PEG hydrogel and protein micropatterns. (A-B) SEM micrographs of hydrogel microstructures fabricated on glass substrates. (C-D) Registration of hydrogel structures, interconnected pads in (C) and rings in (D), with micropatterns of collagen-FITC (green) and collagen-Cy3 (red) respectively. This alignment process allowed to juxtapose cell-adhesive collagen domains with non-fouling PEG regions. (E-F) After incubation with micropatterned surfaces primary rat hepatocytes selectively adhered to collagen domains leaving glass regions open for attachment of “stickier” fibroblasts.

Figure 4.

Formation of micropatterned co-cultures around hydrogel structures. (A-B) Selective attachment of rat hepatocytes to collagen domains located within PEG gel rings (A) and around hydrogel pads (B). (C-D) 3T3 fibroblasts added to the surface attached on silane-modified glass regions next to hepatocytes. PEG hydrogel structures did not support cell attachment during co-culture assembly and remained non-fouling after one week in culture. (E-F) Fluorescent labeling of cells showing distinct population of hepatic (HepG2) cells (green) co-cultured with fibroblasts (red). PEG hydrogel features did not support cell attachment and are non-fluorescent. Hepatocytes could be separated from fibroblasts by PEG hydrogel fence (F), pointing to the possibility of controlling interactions between two cell types.

Figure 5. Integration of enzyme-containing PEG hydrogel structures with micropatterned co-cultures. (A) SEM micrograph of HRP-carrying hydrogel features juxtaposed with cells. Hepatocytes form a dense cell layer next to the hydrogel pad. Elongated fibroblasts reside next to hepatocytes, toward the bottom of the image. (B-C) Merged brightfield/fluorescence images before (B) and after (C) addition of 10 μM H₂O₂. Strong red fluorescence observed in (C) points to retained activity of entrapped HRP.