Non-positional cell microarray prepared by shape-coded polymeric microboards: A new microarray format for multiplex and high throughput cell-based assays

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A non-positional (or suspension) cell microarray was developed using shape-coded SU-8 photoresist microboards for potential application in multiplex and high-throughput cell-based assays. A conventional photolithography process on glass slides produced various shapes of SU-8 micropatterns that had a lateral dimension of 200 μm and a thickness of 40 μm . The resultant micropatterns were detached from the slides by sonication and named “microboards” due to the fact that had a much larger lateral dimension than thickness. The surfaces of the SU-8 microboards were modified with collagen to promote cell adhesion, and it was confirmed that collagen-coated SU-8 microboards supported cell adhesion and proliferation. Seeding of cells into poly(ethylene glycol)(PEG) hydrogel-coated well plates containing collagen-modified microboards resulted in selective cell adhesion onto the microboards due to the non-adhesiveness of PEG hydrogel toward cells, thereby creating non-positional arrays of microboards carrying cells. Finally, two different cell types (fibroblasts and HeLa cells) were separately cultured on different shapes of microboards and subsequently mixed together to create a non-positional cell microarray consisting of multiple cell types where each cell could be easily identified by the shape of the microboard to which they had adhered. Because numerous unique shapes of microboards can be fabricated using this method by simply changing the photomask designs, high throughput and multiplex cell-based assays would be easily achieved with this system in the future.

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I. INTRODUCTION

Cell-based biosensors are devices that use living cells as biorecognition elements to detect a broad range of agents. These devices have been receiving attention recently because of potential applications in high-content drug screening and the detection of biological warfare agents and pathogens.^{1–3} One of the most important reasons for using living cells as sensing elements is that they can provide not only analytical information, which indicates how much of a given substance is present, but also functional information, i.e., information about the effect of an external stimulus induced by a certain substance on a living system.⁴ There are many circumstances in which functional rather than analytical information is ultimately needed, because analytical information is usually necessary to estimate the functional consequences of a substance. Although cell-based systems often exhibit a long response time and poor selectivity, they have several advantages over other biosensors that are based on enzymes or antibodies, including extremely high sensitivity, the ability to detect a large number of analytes, and the ease of growing and isolating sensing components (i.e., the cells).^{5,6} Among various applications, one particularly important application is drug screening because cells represent the ultimate target

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for pharmaceuticals.⁷ Cell-based assays have played important roles in evaluating potential drug targets by functionally characterizing their effect on cells, assessing the specificity and efficacy of drug leads, and identifying the targets for drugs with an unknown mechanism of action. Over the past decade, a variety of scientific advances that demand the synthesis of a large number of drug candidates in addition to the economic pressure to reduce development costs have driven the need for high-throughput cell-based assays using miniaturized multiplexed assay formats.^{8–12} Assay throughput was initially addressed by growing cells in 96-, 384-, or 1536-well microplate format. However, there are still strong demands to further miniaturize cell-based assays to increase the parallelism of cell analysis.^{13–16} To that end, cell microarrays provide an attractive solution; thousands of cells can be immobilized either on a planar surface (positional array) or on microparticles (non-positional array or suspension array) to realize multiplexed and high-throughput assays.^{17,18} Positional arrays have been generated by printing cells on different microdomains within a plane surface, such as a glass slide. In the last decade, significant efforts have focused on the fabrication of positional cell microarrays using a variety of patterning techniques such as spotting, photolithography or soft lithography.^{19–26} A positional array can contain at least 5000–6000 spots of cells on one glass slide, and each cell is identified by *x* and *y* coordinates on the microarray. Although positional arrays have been well publicized and widely used for various applications requiring ultra-high-density analysis, non-positional arrays are becoming popular as an alternative approach for high-throughput assays because it is believed that they have greater flexibility for multiplex assays.^{27–31} In the case of positional microarrays, patterning of different cells on single substrates is difficult and requires a complex process. However, with non-positional arrays, multiplex assay systems can be easily achieved by simply mixing the independently prepared microparticles carrying different cells. In this case, the identity of the cells is determined by self-encoded microparticles that carry the cells as array elements, instead of positionally encoded spots on a planar surface. Various strategies have been investigated to encode microparticles such as optical, electronic, photophysical, and graphical encoding.^{32–37} However, most of these methods required complicated coding and decoding processes that use expensive equipment. Furthermore, applications of non-positional arrays have been mostly focused on multiplex protein- and nucleic acid-based assays, and there are only a few reports that deal with cell microarrays using encoded microcarriers.

In this study, we prepared a shape-coded non-positional cell microarray using SU-8 photoresist. Photopatterning and a subsequent sonication step produced various shapes of SU-8 microparticles, which we named microboards due to their greater lateral dimension than thickness. Unlike that of other coding methods, shape coding can be easily accomplished using photolithography that can produce a large number of unique shapes, and decoding can be done using optical microscopy. After the suitability of SU-8 microboards for cell adhesion and growth was confirmed, a shape-coded non-positional cell microarray, in which different cells were immobilized onto differently shaped microboards, was prepared for potential application in a multiplex cell-based assay.

II. MATERIALS AND METHODS

A. Materials

SU-8 50 negative photoresist and developer solution were obtained from Microlithography Chemical Corp. (Newton, MA). Murine 3T3 fibroblast and HeLa cells (human epithelial carcinoma cell line) were obtained from American Type Culture Collection (Manassas, VA). For the cell culture, [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT)], Dulbecco's modified Eagle's medium (DMEM), collagen type I from human skin, L-glutamine, antibiotic/antimycotic solution, trypsin, fetal bovine serum (FBS), and ethylenediaminetetra-acetate (EDTA) were purchased from Sigma-Aldrich (Milwaukee, WI, USA). Poly(ethylene glycol) diacrylate (PEG-DA) (MW 575) and 2-hydroxy-2-methylpropiophenone (HOMPP) were also obtained from Sigma-Aldrich, and disk-type PEG hydrogels were prepared as previously described.³⁸ Calcein-AM and CellTracker Orange CMTMR [5-(and-6)-(((4-chloromethyl)benzoyl)amino)tetramethylrhodamine] were purchased from Invitrogen (Carlsbad, CA, USA), and

were used to obtain fluorescence images of adherent cells. Photomasks for photolithography were prepared using AUTO CAD and printed on transparencies using a standard laser jet printer (LaserWriter 16/600 PS, Apple, Inc., Cupertino, CA, USA). Phosphate buffered saline (PBS, 0.1 M, pH 7.4) consisted of 1.1 mM potassium phosphate monobasic, 3 mM sodium phosphate dibasic heptahydrate, and 0.15 M NaCl in deionized water.

B. Preparation of microboards

SU-8 50 photoresist was used to produce microboards. First, SU-8 50 photoresist was spin-coated on bare glass slides for 20 s at 2000 rpm then baked for 20 min at 95 °C on a horizontal hot plate (soft bake). Photoresist-coated glass slides were then exposed to 365 nm UV light through the photomasks, followed by a post bake at 95 °C for 15 min. Desired micropatterns were obtained by dissolving away the unpolymerized photoresist using developer solution. The resultant micropattern-containing glass slides were immersed in ethyl alcohol and sonicated for 1 h, which detached the micropatterns from the glass slide, thus producing microboards.

C. Cell culture

Murine fibroblasts and HeLa cells were cultured in DMEM supplemented with FBS (10%), and L-glutamine (584 mg/L), penicillin (100 µg/mL), and streptomycin (100 µg/mL). Both phenotypes were grown to confluence in 75 cm² polystyrene tissue culture flasks and confluent cells were subcultured every 2–3 days by trypsinization with 0.25% (w/v) trypsin and 0.13% (w/v) EDTA. All cell lines were incubated at 37 °C in 5% CO₂ and 95% air.

D. Cell culture on microboards

Prior to cell seeding, microboards were coated with collagen to facilitate cell adhesion as described previously.³⁹ Briefly, microboards were pretreated with 100 µg/mL poly(D-lysine) in PBS for 16 h to facilitate collagen adsorption and then immersed in a solution of collagen (400 µg/mL) in acetic acid (2 mM). After collagen immobilization, microboards were washed extensively with PBS until no collagen was observed in the washing solution. Collagen-coated microboards were transferred to 12-well plates that were covered with PEG hydrogel disks (about 360 microboards per each well). To seed cells into microboard-containing wells, cells were trypsinized from routine culture and centrifuged. The supernatant was removed, and the cells were resuspended in fresh culture medium without FBS. An aliquot was obtained for cell counting in a hemocytometer to adjust seeding density. Finally, the desired amount of cells was seeded into each well. After incubation for 4 h, culture medium containing floating cells was removed from the well plates and replaced with fresh culture medium. To prevent removal of microboards during exchange of culture medium, all the microboards were placed on one side of culture wells using cell scraper and media was aspirated at the opposite side of culture wells that were tilted to the same direction. The overall process to obtain cell-attached microboards was described in Figure 1. Cell adhesion and proliferation on the SU-8 surfaces was investigated by using the MTT assay and counting the number of cells on the surfaces. To obtain fluorescence images of the cells, 10 µM of Calcein-AM or CellTracker Orange CMTMR was prepared by diluting the stock solution dissolved in anhydrous dimethyl sulfoxide (DMSO) with cell culture media. Cells were incubated with these solutions for 30 min. and then observed with a fluorescence microscope. All the cell experiments with microboards were performed under static condition and all the microboards maintained their original shapes throughout the experiments.

E. Image acquisition

Adhered cells on microboards were fixed with 4.0% glutaraldehyde at 4 °C for 4 h for imaging by optical/fluorescence microscopy and scanning electron microscopy (SEM). A Zeiss Axiovert 200 microscope equipped with an integrated color CCD camera (Carl Zeiss Inc., Thornwood, NY, USA) was used to obtain optical and fluorescence images, while SEM was performed with a JEOL T330A at 15 kV (JEOL, Ltd., Peabody, MA, USA).

FIG. 1. Schematic illustration of preparing a non-positional (or suspension) cell microarray using SU-8 microboards.

III. RESULTS AND DISCUSSION

To prepare the different shapes of microboards, SU-8 photoresist was first photolithographically patterned using the photomasks. SU-8 is an epoxy-based negative photoresist, which is a mixture of EPON SU-8 resin, a solvent (either γ -butyrolactone or cyclopentane) and a photoacid generator (a triarylsulfonium hexafluoroantimonate salt).⁴⁰ After the viscous SU-8 resin was spin-coated onto a glass slide, the solvent was evaporated during a soft bake step. Upon exposure to UV (350~400 nm) light, cross-linking proceeded in two steps: (1) formation of a strong acid during the exposure process, followed by (2) acid-initiated, thermally driven epoxy cross-linking during the post-exposure bake step. Therefore, when the SU-8 coated glass slides were exposed to UV light through the photomasks, only the exposed region underwent cross-linking and became insoluble in the liquid developer, thus creating desired patterns on the glass slides. Figure 2(a) shows the different shapes of SU-8 micropatterns fabricated using simple photolithography. The lateral dimension and shape of the micropatterns was controlled by the design of the photomask, while the height was dependent on the thickness of the photoresist. In this study, we fabricated SU-8 micropatterns using photomasks containing arrays of circles, squares, and triangles with a lateral dimension of 200 μm . The resultant micropatterns were effectively released from the glass slides via sonication with about 80% releasing efficiency, producing approximately 40- μm -thick microboards of different shapes [Figure 2(b)]. High magnification SEM images revealed that surfaces of the microboards were very smooth and lacked serious defects [Figure 2(c)]. The ability to control the shapes of the cell microcarriers allows for the production of shape-coded non-positional arrays, which can offer a cost-effective and versatile platform for high-throughput and multiplexed bioassays. Currently, most non-positional array systems use color-coded microparticles in multiplexed assays. However, there are several disadvantages to using multiple colors as a means of encoding in cell microarrays including the limited number of color combinations and spectral overlap between the encoding color and the color used for analyte detection or the fluorescent staining of cells. Furthermore, decoding requires expensive fluorescence equipment. Shape-coded non-positional arrays are expected to overcome the problems mentioned above, since a large number of unique shapes are possible by changing the designs of photomasks, and decoding can be easily performed using optical microscopy to verify the shapes of the microboards.

FIG. 2. SEM images of SU-8 microboards prepared by photopatterning and subsequent sonication. (a) Different shapes of SU-8 micropatterns on glass slides. (b) SU-8 microboards prepared by detaching micropatterns from glass by sonication. (c) High magnification SEM images of microboards.

Prior to cell seeding onto the microboards, we first investigated cell adhesion and growth on the surface of the SU-8 photoresist. Because it was reported that most cells would not attach to or grow on native SU-8 due to its hydrophobicity,^{41,42} the surface of the SU-8 was modified with collagen to enhance cell adhesion. The presence of collagen on the SU-8 surface was confirmed by XPS (x-ray photoelectron spectroscopy), as shown in Figure 3. New N1s signals that were not present for the bare SU-8 were observed after collagen modification, which is indicative of peptide linkage on the SU-8 surface. When fibroblasts were seeded onto the collagen-modified SU-8, they were distributed homogeneously over the entire substrate, and overall cell morphology was comparable to cells cultured in normal tissue culture flasks [Figure 4(a)]. Because of the transparent nature of SU-8, adherent cells could be easily monitored with optical microscopy. The adhered and proliferated cells were first quantified by counting the cells on the SU-8 surfaces over 5 days. Figure 4(b) shows that cells could attach and proliferate over entire culture periods (5 days). The number of adhered cells kept increasing up to 5 days. Cell viability and proliferation on the collagen-modified SU-8 was further investigated using the MTT assay. The MTT assay provides an indirect estimation of the activity of mitochondrial oxidative processes in living cells. Various dehydrogenase enzymes in active mitochondria are able to cleave MTT, a pale yellow substance, and convert it to purple formazan crystals. Here, colorimetric measurement of formazan dye was performed on a spectrophotometer with an optical density reading at 490 nm, where the absorbance value is proportional to the number of cells that remain viable. An increase in absorbance with culture time demonstrated that the collagen-modified SU-8 supported cell proliferation [Figure 4(c)], which further supports the cell counting results. Similar results were obtained with HeLa cells.

FIG. 3. N1s XPS spectra of SU-8 surface before and after collagen modification

After confirming that collagen-coated SU-8 could support cell adhesion without losing viability, cells were seeded onto PEG hydrogel-covered 12-well plates that contained collagen-coated SU-8 microboards. If the well plates were not covered with PEG hydrogels, more cells tended to adhere onto the well plates or some cells adhered onto both the microboards and well

(a)

(b)

(c)

FIG. 4. Cell culture on collagen-coated SU-8. (a) Optical and fluorescent images of fibroblasts after 1 day of culture. (b) Evaluation of fibroblast counts. (1.0×10^4 cells were seeded onto a SU-8 substrate). (c) Change of absorbance resulting solution from MTT assay on fibroblasts.

FIG. 5. Cell-adhered SU-8 microboards. Optical and fluorescent images of fibroblasts that adhered on (a) square-shaped and (b) circle-shaped microboards (scale bar: 200 μm). (c) SEM images of adhered fibroblasts on microboards.

plates; the former produced many microboards without cells and the latter made it difficult to detach cell-containing microboards from the well plates later. Figure 5(a) shows the optical and fluorescent images of the square-shaped microboards that were incubated with fibroblasts for 10 h. Because of non-adhesiveness of PEG hydrogel toward cells, most of adherent cells were found only on the microboards after culture medium containing floating cells was replaced with fresh medium. The fluorescent images of the cells were obtained with Calcein-AM. Calcein-AM is colorless and nonfluorescent compound that can permeate cell membranes. Once inside

FIG. 6. (a) Average number of cells in one microboard and (b) cell occupancy of microboards as a function of cell seeding density. Circle-shaped microboards were used.

the cells, the ester groups of the Calcein-AM compound are hydrolyzed by cellular esterase enzymes to yield calcein, which is fluorescent and emits green light. The strong green emitted light, shown in Figure 5(a), confirms that the cells on the microboards maintained their viability and enzyme activity. Similar results were obtained from circle-shaped microboards [Figure 5(b)]. SEM images revealed that adhered fibroblasts dispersed sufficiently on the surface of the microboards and maintained normal morphology [Figure 5(c)]. Cell occupancy of the microboards (microboards containing adhered cells/total number of microboards) and the average number of cells per one microboard were dependent on the cell seeding density, as shown in Figures 6(a) and 6(b), respectively. Although the trends shown in these figures indicate that both cell occupancy and the number of cells increased with cell seeding density, precise control was difficult and resulted in a large standard deviation. When 10^6 cells were seeded into well

FIG. 7. Effect of longer cell culture periods on suspension cell microarray. (a) Optical and fluorescent images of cell-adhered microboards where cells on different microboards connected by proliferation (b) Local aggregation of microboards induced by the connections between the cells on different microboards.

FIG. 8. A non-positional microarray of microboards that contained multiple cell types (fibroblasts and HeLa cells). Fibroblasts and HeLa cells were stained green and orange, respectively, and each cell was also identified by shape of microboards.

plates that contained circle-shaped microboards, about 80% of the microboards were occupied with cells and about averagely 50 cells adhered onto one microboard. Interestingly, as culture periods were prolonged (after 3 days), the cells on a microboard proliferated and connected to cells on other nearby microboards [Figure 7(a)]. The connections between the cells on different microboards induced aggregation of the microboards as shown in Figure 7(b). The microboard aggregation could be avoided by using less microboards per well, such that direct contact between microboards would not occur.

Finally, we demonstrated the possibility of preparing shape-coded non-positional arrays containing different cell types for the potential application as a multiplex cell-based assay system. Since a lot of unique microboard shapes can be prepared by this method, non-positional arrays of multiple cell types can be prepared by randomly assembling a mixture of the desired non-positional arrays. To verify this concept, we used fibroblasts and HeLa cells stained with Calcein AM and Cell Tracker Orange, respectively. Square- and circle-shaped microboards were incubated with fibroblasts and HeLa cells independently in different well plates. After cell adhesion, these different microboards were transferred to a single well and mixed, creating a non-positional array of microboards that contained multiple cell types (Figure 8). Although two differently colored fluorescent molecules were used to more clearly visualize and identify each cell in this study, staining or other color coding is not necessary in our shape-coded non-positional array system because the shape of the microboards alone is sufficient to identify the adhered cells.

IV. CONCLUSION

In this study, we fabricated differently shaped polymeric microboards in order to create a shape-coded non-positional array of cells. To prepare the microboards, micropatterns of SU-8 photoreist were first fabricated on glass slides using conventional photolithography. Detachment of the SU-8 patterns using sonication produced the desired microboards with sizes and shapes that were controlled by the design of the photomasks. The collagen-modified SU-8 could support cell adhesion and proliferation. Cell seeding into microboard-containing well plates covered with PEG hydrogel resulted in the selective adhesion of cells onto the microboards due to the lack of affinity between PEG hydrogel and the cells; this successful selective adhesion created non-positional arrays of cell-containing microboards. Cell occupancy of the microboards and the average number of cells per one microboard could be controlled by changing the cell seeding density. Finally, a mixture comprised of different cell types adhered to two different microboard shapes was prepared to prove that our non-positional arrays can be used as a multiplexed cell-based assay system. In the future, the microboards presented herein can be

combined with a microfluidic device to create a multi-analyte cell-based biosensor in a lab-on-a-chip format.

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