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SU-8 microstructure for quasi-three-dimensional cell-based biosensing

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ABSTRACT

A quasi-three-dimensional (quasi-3-D) cell-based biosensor platform microfabricated from SU-8 has been developed and characterized. In this work, SH-SY5Y human neuroblastoma cells were integrated with SU-8 microfabricated microwells with diameters of 100 μm. SH-SY5Y cells were differentiated with 1 mM dibutyryl cAMP and 2.5 μM 5-bromodeoxyuridine. Voltage-gated calcium channel (VGCC) function of SH-SY5Y cells cultured within the microwells (quasi-3-D) versus those cultured on the SU-8 planar substrates (2-D) was evaluated by confocal microscopy with a calcium fluorescent indicator, Calcium Green-1. In response to 50 mM high K^+ depolarization, cells in microwells were less responsive in terms of increase in intracellular Ca^{2+} in comparison to cells on 2-D substrates. This study shows that VGCC function of cells within SU-8 microwells was indeed different from that of cells on planar SU-8 surfaces, suggesting that SU-8 microstructure did affect SH-SY5Y cell differentiation with respect to VGCC function and that high-aspect-ratio microstructures are not merely "folded" 2-D structures. Furthermore, these results are consistent with previous 2-D/3-D comparative studies carried out in polymer scaffolds and support the hypothesis that cell calcium dynamics on 2-D substrates may be exaggerated. Overall, this work is supportive of SU-8 micropattern as a viable platform for engineering a quasi-3-D cell culture system for cell-based biosensing against drugs for VGCCs.

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1. Introduction

Unlike molecular biosensors which use isolated enzymes, antibodies or nucleic acids, cell-based biosensors employ the whole cell as the sensing element and thus can provide information in terms of whole cell response [1]. In accelerated drug discovery, cell-based biosensors play an important role by providing functional and analytical information which is more accurate and physiologically relevant than what molecular biosensors can offer [2,3]. Thus, a cell-based assay can well bridge the gap between pure in vitro systems and in vivo test. With the development of fluorescence based technologies and their implementation in high-throughput screening (HTS) systems (e.g., fluorometric imaging plate reader (FLIPR) molecular device), cellular assay based on fluorescence imaging has proven to be an invaluable tool in drug discovery. However, in the application of cell-based biosensors, cells are cultured on two-dimensional (2-D) flat surfaces [4,5], which is contrary to how cells grow in vivo where extracellular matrix provides a three-dimensional (3-D) environment. This raises the issue of in vivo–in vitro differences from cell morphology to cell functions and further to gene expression. It has been hypothesized that cell function on 2-D surfaces may be an exaggeration of that in vivo [6], which necessitates investigating 3-D cell culture systems [7] and calls for 2-D/3-D comparative study for the purpose of biosensor application [8,9].

Polymers, such as collagen hydregels [10–12], N-(2-hydroxypropyl)-methacrylamide (HPMA) copolymer hydrogels [13], poly(lactic-co-glycolic acid) (PLGA), and poly(L-lactic acid) (PLLA) [14], have been applied as scaffolds to study cell growth, proliferation, differentiation as well as cell function in 3-D environments. To apply cell-based biosensors in screening drugs against voltage-gated calcium channels (VGCCs), Mao and Kisaalita [8] and Desai et al. [9] conducted comparative studies of VGCC function of cells in 3-D collagen and on 2-D surfaces and their results showed 2-D/3-D differences with respect to cell function. However, these polymers have several limitations. First, it is difficult to guarantee precise control of the aspect ratio (height over width) with these structures. Second, limited mass transportation in scaffolds affects nutrient supply, waste drainage and drug exposure. Third, the properties of polymer scaffolds may limit the extent to which optical or electrical detection may be applicable.

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Microfabrication, which can produce a miniaturized, inexpensive platform for "cell-culture-on-a-chip", can be an ideal way to engineer a quasi-3-D environment for cell culture [15]. In comparison with other 3-D cell culture systems formed from polymers, microfabricated patterns have many advantages. First, the technique has been well developed. The aspect ratio can be well controlled in fabrication process and the fabrication of microstructures can be scaled up to meet the need of HTS. Second, cells can be easily seeded in these patterns and integrated with optical as well as bioelectrical analytical methods [16,17]. Third, unlike cells embedded in collagen, cells cultured in microstructures are better exposed to growth media and drug supply; thus the mass transfer limitation problem can be minimized in the microfabricated patterns, which is an essential aspect in HTS. In addition, these platforms can allow precise control of the environment surrounding individual cells and they have been used to study physiologic and pharmacologic responses at the single-cell level. On-chip individual-cell-based cultivation system from Yasuda's lab has been reported to study single cell growth and division dynamics [18] as well as cell-to-cell communication effects in cell group class [19]. Microfabricated structures are gaining popularity for studies of microbiology [20] and cellular biology [21–25]. They have become powerful platforms for studying cell fate in vitro [26,27]. However, most of these microstructures are low-aspect ratio, which have been considered as 2-D systems. Thus we proposed to fabricate high-aspect-ratio microstructures to mimic 3-D microenvironments for cell integration.

In this study, SU-8 was chosen to fabricate microwells with diameters of 100 μm. These micropatterns provided high-aspect-ratio structures for cell culture. SU-8, which is epoxy based negative photoresist, can form a film with a thickness from 1 μm to 200 μm with the spin coating processes. Films can be processed with photolithography to pattern high-aspect-ratio (>20) structures with high optical transparency, straight sidewalls, and excellent thermal stability. SU-8 is wildly used in fabrication of microfluidics and microelectromechanical system (MEMS) parts [28–30]. Also, SU-8 microstructure is considered a novel platform for integrating with cell culture and studying cell fate in vitro [22,31]. In the present work, SH-SY5Y human neuroblastoma cells were integrated with SU-8 microwells and differentiated with 1 mM dibutyryl cAMP and 2.5 μM 5-bromodeoxyuridine. Because of their importance in the central nervous and cardiovascular systems, VGCCs are becoming popular drug targets in the drug discovery. The measurement of intracellular calcium fluxes in real time is widely applied within the pharmaceutical industry to measure the activation of VGCCs. For the development of a cell-based biosensor toward screening drugs against VGCC targets, we evaluated VGCC response of SH-SY5Y cells by confocal microscopy with a calcium fluorescent indicator, Calcium Green-1. Intracellular Ca^{2+} dynamics of SH-SY5Y cells cultured in microwells versus on 2-D substrates as a control group were compared. The goal was to explore the effects of the microstructure on cell function with respect to VGCC functionality and provide a proof-of-concept for quasi-3-D cell-based assay for VGCCs.

2. Materials and methods

2.1. Fabrication of SU-8 microstructure

Flat SU-8 substrates and microwell structures were fabricated on 25-mm coverslips (Fisher Scientific, Pittsburgh, PA, USA). Before fabrication, the coverslips were cleaned with 20% sulfuric acid and then baked at 110 °C for at least 3 h. SU-8 (2025, MicroChem, Newton, MA, USA) was spun onto the glass substrate at a speed of 1000 rpm for 30 s. 25% (w/v) photoresist was used to achieve flat SU-8 surfaces, and the coating thickness was approximately 1.5 μm [15]. 69% (w/v) SU-8 was used for fabricating microwell structures and the resulting coating thickness was about 70–150 μm [15], depending on the processing conditions. Then the SU-8 coated coverslips were soft baked, first at 65 °C for 3 min and then at 95 °C for up to 30 min. After baking, SU-8 was exposed in soft contact mode with a Karl Suss MJB 3 HP Mask Aligner using 365 nm UV at 10 mW for four successive 15 s, interrupted for at least 20 s. No mask was used to expose the whole surface area

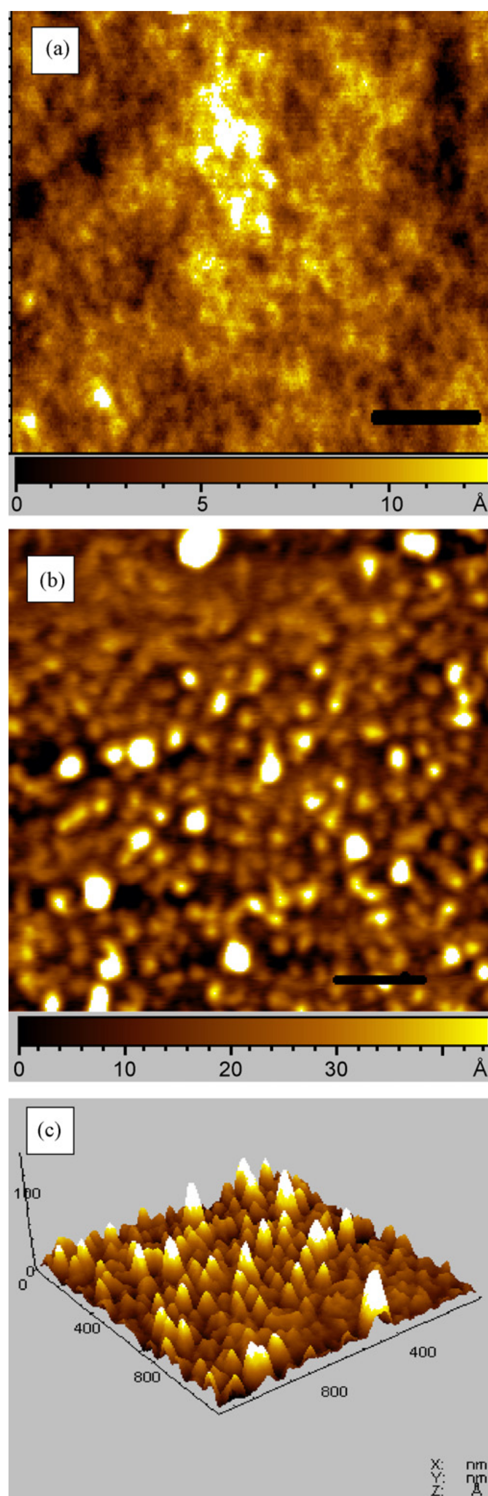

Fig. 1. AFM images of SU-8 surface (a) and SU-8 surface coated with poly-L-lysine (b) with its geometric structure (c). In (c), the units for X and Y axes are in nanometers, while for Z axis the units are in anstrons. Bar = 200 nm.

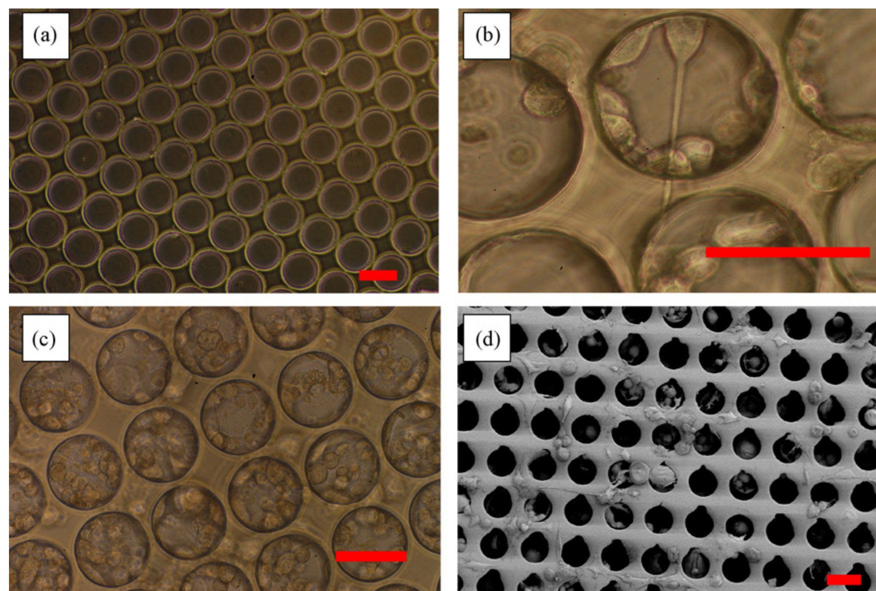

Fig. 2. Phase contrast and SEM images of 100 μm SU-8 microwell patterns: (a) phase contrast image of patterns without cells; (b) phase contrast image of patterns with SH-SY5Y cells on day 8 into differentiation; (c) phase contrast image of pattern with SH-SY5Y cells on day 2 into differentiation; (d) SEM micrograph of patterns with SY-SY5Y cells on day 2 into differentiation. Bar = 100 μm.

of the flat SU-8 substrates while a chromium mask was used to fabricate the microwell patterns. Patterns and their nominal structure dimensions used in this study included 100 μm wells with a center-to-center spacing of 110 μm, and 100 μm wells with 10 μm wide channel connection. After exposure, the SU-8 coating was baked again, first at 65 °C for 3 min and then at 95 °C for 9 min before development. Patterns were developed with SU-8 developer (MicroChem, Newton, MA) for 14 min and then briefly immersed in isopropyl alcohol (Fisher Chemicals, Fairlawn, NJ) before drying with nitrogen. Finally, the patterns were hard baked at 120 °C for at least 30 min. Samples were sterilized by immersion in 70% ethanol in distilled water (DI) under a UV germicidal lamp overnight, rinsed in sterile DI water 3 times and stored wet until need.

2.2. Atomic force microscopy

To characterize SU-8 surface topography and confirm the poly-L-lysine coating on the substrates, we used a PicoPlus-SPM (Agilent-Molecular Imaging) system for AFM imaging of the planar SU-8 surface itself as well as the bulk region of poly-L-lysine coating of the 2-D substrates. Silicon tips were used. Acoustic AC mode (AAC) was applied to obtain high resolution topographic images to overcome problems due to friction, adhesion and electrostatic forces that are normally experienced with Contact mode AFM. In AAC mode, cantilever oscillation is accomplished by indirect vibration, in which the cantilever is excited by high frequency acoustic vibration from a piezoelectric transducer attached to the cantilever holder. Tip-sample force interactions cause changes in amplitude, phase and the resonance frequency.

2.3. Cell line and cell culture

SH-SY5Y human neuroblastoma cells were obtained from ATCC and routinely cultured in 75 cm^2 tissue culture flasks (Costar, Corning, NY) with the growth medium in a 10% CO_2 humidified air at 37 °C. The growth medium was made with Eagle Minimum Essential Medium (MEM) containing 10% heat inactivated fetal bovine serum (FBS), 2.2 g/L sodium bicarbonate, 2 mM L-glutamine and 1 mM sodium pyruvate [8,9]. At 75% confluence, the cells were detached by mechanically pipetting and re-suspended in growth medium for plating. Before plating, SU-8 microwell patterns and flat substrates were coated with poly-L-lysine for at least 1 h to enhance the cell attachment. Then these flat substrates and microstructure pattens were washed with growth media twice. Approximately 5×10^5 cells were plated on each patterned substrate or flat substrate in growth medium, which was contained in a 35 mm Petri dish (FALCON, Becton Dickinson Labware, NJ). On the second day after plating, referred to as day 0 into differentiation hereafter, the medium was changed from growth medium to differentiation medium. The differentiation medium was comprised of MEM with 5% FBS, 2.2 g/L sodium bicarbonate, 2 mM L-glutamine, 1 mM sodium pyruvate, 1 mM dibutyrylcAMP (dcAMP) and 2.5 μM 5-bromodeoxyuridine (BrdU) [8,9]. Differentiation medium was changed daily.

2.4. Fluorescence staining and fluorescent microscopy

SH-SY5Y cells were stained with calcein AM (Biotium, Hayward, CA). This fluorescent dye stains living cells and their extensions by the presence of intracellular esterase activity, which converts the non-fluorescent cell-permeant calcein AM to intensely fluorescent calcein. Cells were washed with 2 mL phosphate-buffered saline (PBS) three to five times. Then cells were exposed to 2 ml 2-μM calcein AM in PBS and incubated for 30 min at 37 °C before the staining solution was replaced. Samples were washed with PBS 3 times. Sample fluorescence was captured with a B-2E/C FITC filter block (Nikon, Melville, NY, USA), which has an excitation bandwidth of 465–495 nm and a filter pass range of 515–555 nm.

2.5. Scanning electron microscopy (SEM)

Cells on SU-8 patterns were fixed with 2% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.2 for 1 h before rinsing in cacodylate buffer (without glutaraldehyde) three times, 15 min each. This was followed by post-fixing with 1% OsO_4 in 0.1 M sodium

cacodylate buffer for 1 h and rinsing in cacodylate buffer (without OsO4) three times, 5 min each. The samples were then dehydrated successively in 30%, 50%, 70%, 80%, 95% and 100% ethanol for 10 min each and dried in a SAMDRI-780A critical point drier (Tousimis Research Corporation, Rockville, MD, USA). Patterns were sputter-coated with gold for 60 s to achieve a coating thickness of about 15.3 nm. SEM images were captured with LEO 982 scanning electron microscope (LEO Electronenmikroskopie GmbH Korporation, Germany) with an acceleration voltage of 5 kV, using either the regular detector or in-lens detector.

2.6. Evaluation of the VGCC functionality

VGCC functionality was evaluated with the dynamics of calcium influx in response to high K^+ (50 mM) depolarization. The membrane permeable fluorescent dye, Calcium Green-1, acetoxymethylester (AM) (Molecular Probes, Eugene, OR), was used to visualize the calcium influx dynamics. The fluorescent intensity was measured by a confocal imaging system (PCM-2000, Nikon) linked to an inverted (TE300, Nikon) microscopy and a 60× Aprochromat, oil-immersion, high-numerical aperture (1.4) objective lens. On days 2 and 8 into differentiation, cells on either flat substrates or in microwell structures were washed with HBS twice and loaded with 5 μM Calcium Green-1 AM in 1 ml of HBS containing 3% heat inactivated FBS and 0.02% Pluronic F-127 (Molecular Probes, Eugene, OR) for 1 h at 37 °C in a 10% CO_2 humidified incubator. Cells were then washed twice with HBS and incubated with 1 ml HBS at 37 °C in the 10% CO_2 humidified incubator for another hour to allow complete dye de-esterification. Calcium Green-1 was excited with 488 nm argon laser and the emission was captured through a 515 nm long-pass filter. Confocal images were continuously captured at a rate of 1 frame per 3 s. While images were being captured, cells were depolarized by adding 100 μl of high K^+ HBS (500 mM) to achieve a final K^+ concentration of 50 mM (along with 130 mM Na^+ in HBS). Functional VGCCs were demonstrated by cytosolic calcium concentration increase upon depolarization. This intracellular calcium dynamics was reflected by changes in relative intracellular Calcium Green-1 fluorescence intensities, which were plotted as average gray level units.

2.7. Data analysis and statistics

Student's *t*-test was used for statistical comparisons of calcium response magnitudes.

3. Results and discussion

3.1. SU-8 structures for culturing cells

In the present study, planar SU-8 substrates were used to culture SH-SY5Y cells in monolayer, as a 2-D control for the SU-8 microwells. Both the planar surfaces and the microwells were coated with poly-L-lysine. Fig. 1(a) shows an AFM image of the planar SU-8 surface. The surface is smooth and no geometric structures can be observed. Poly-L-lysine has been applied to patten silicon surface for directing neural cell growth [32] and deposization of poly-L-lysine has been observed on mica surface with Au-nanoparticles [33]. Fig. 1(b) shows an AFM image of the bulk region of poly-L-lysine coating of the 2-D substrates. This image shows that poly-L-lysine covered the substrate homogeneously and uniformly with a felt-like structure of deposited molecules. Detailed structure of coating can be viewed from the geometric coating image (Fig. 1 (c)). In Fig. 1 (c), poly-L-lysine molecule nanoparticles randomly deposited on the substrate.

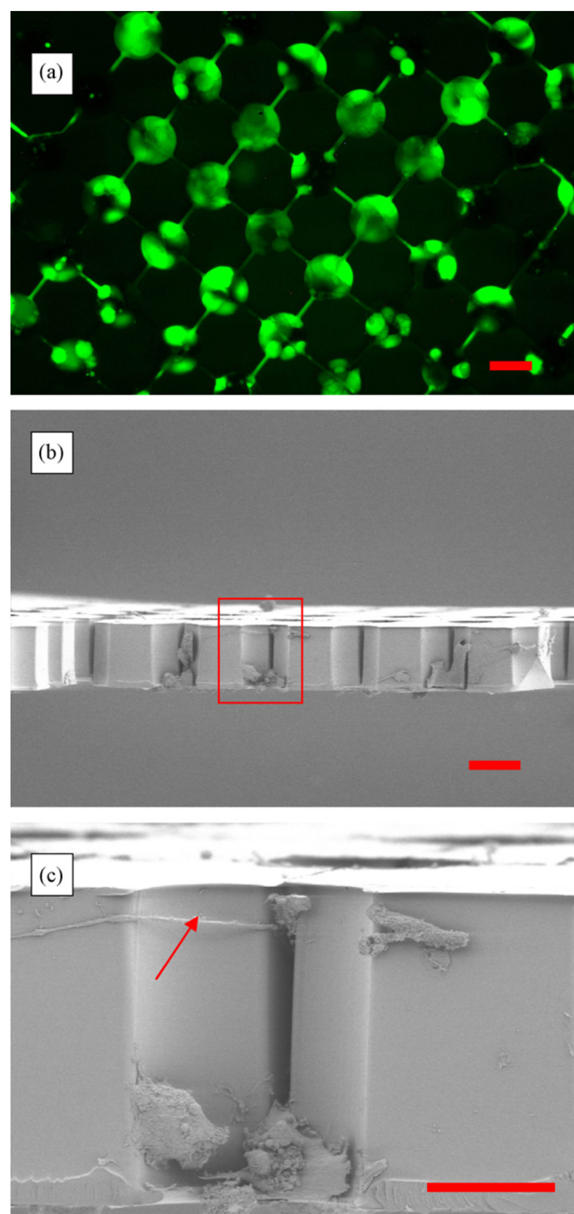

Fig. 3. Images of SH-SY5Y cells in network micropatterns on day 8 into differentiation: (a) calcein stained cells; (b) section of patterns; (c) blow-up of showing neurite from adjacent well (red arrow) and cell cluster at the bottom of the well. Bar = 100 μm in (a) and (b), bar = 50 μm in (c).

For SH-SY5Y cell integration, SU-8 patterns with a diameter of 100 μm were used in this study. Fig. 2(a) shows the representative microwell structure without channels and Fig. 2(b) shows SH-SY5Y cells cultured in a 100 μm (diameter) pattern on day 8 into differentiation. Fig. 2(c) and (d) showed SH-SY5Y cells in microwells on day 2 into differentiation. Cells attached to the sidewalls, and they formed cellular clusters in the wells. In the phase contrast images (Fig. 2(b) and 2 (c)), some of the cells seem out-of-focus because they were not in the same plane; instead, they were distributed in the 3-D space. These observations were confirmed by SEM in Fig. 3(b) and (c). For some cells, neurite outgrowth can be observed. The neurite shown in Fig. 2(b) was from one cell which grew in the well, and it extended to the adjacent well. From the network-like cell patterning (Fig. 3(a)) in microwell structures with channels, neurites were observed along the channels (Fig. 3(c)).

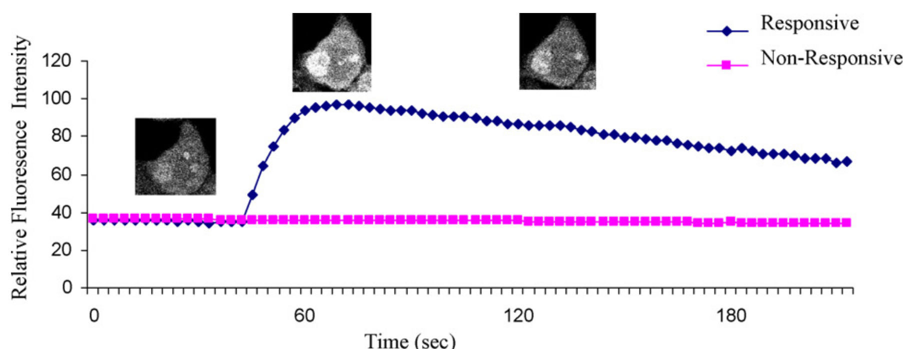

Fig. 4. High K^+ evoked calcium transients in SH-SY5Y cells. Typical plot of changes in relative intracellular Calcium Green-1 fluorescence intensities for responsive and non-responsive cells on 2-D SU-8 substrates at day 2 into differentiation. The responsive cell was from the data pool of 67 cells measured in Table 1. Inset: Confocal images of the cell on 2-D SU-8 substrates at day 2 into differentiation. Images show fluorescence intensity changes before depolarization, 20 s and 100 s after depolarization.

3.2. VGCC responsiveness evaluation

We evaluated the VGCC function of SH-SY5Y cells cultured in microwells and on 2-D substrate with the dynamics of calcium influx in response to high K^+ (50 mM) depolarization. The membrane permeable fluorescent dye, Calcium Green-1, acetoxymethylester (AM) was used to visualize the calcium influx dynamics. Functional VGCCs were demonstrated by cytosolic calcium concentration increase upon depolarization. This intracellular calcium dynamics was reflected by changes in relative intracellular Calcium Green-1 fluorescence intensities, which were plotted as average gray level units. Fig. 4 shows a typical cell and time course of the change in Calcium Green-1 fluorescent intensity for responsive and non-responsive cells upon stimulation with high K^+. The insets are confocal images showing the fluorescence changes in the stimulation process. Fig. 5 shows representative plots of response to high K^+ stimulation on day 8 into differentiation for cells cultured on 2-D substrates (Fig. 5(a)) versus cells in microwells (Fig. 5(b)). Fig. 5 qualitatively shows that cells on 2-D substrates had a larger response magnitude than cells in microwells.

A cell was only considered responsive when it showed an increase in Calcium Green-1 fluorescent intensity of 15% or higher over the basal fluorescent intensity level. The magnitude of the response from each cell was expressed as a peak fractional increase over basal fluorescence intensity. Table 1 summarizes the percentage of responsive cells and magnitudes of VGCC responsiveness (intracellular Ca^{2+} increase) to high K^+ HBS in SH-SY5Y cells cultured in microwells versus on 2-D substrates. As shown in Table 1, it is clear that 100% of the cells on 2-D SU-8 substrates were responsive to high K^+ HBS on days 2 and 8 into differentiation. For cells in microwells, the percentage of responsive cells was 35% on day 2 and decrease to 31% on day 8. Also, cells on 2-D substrates had a larger VGCC response magnitude than cells in microwells on both days 2 and 8 into differentiation ($p < 0.05$). As shown, cells on 2-D substrates had a response magnitude of approximately 1. For

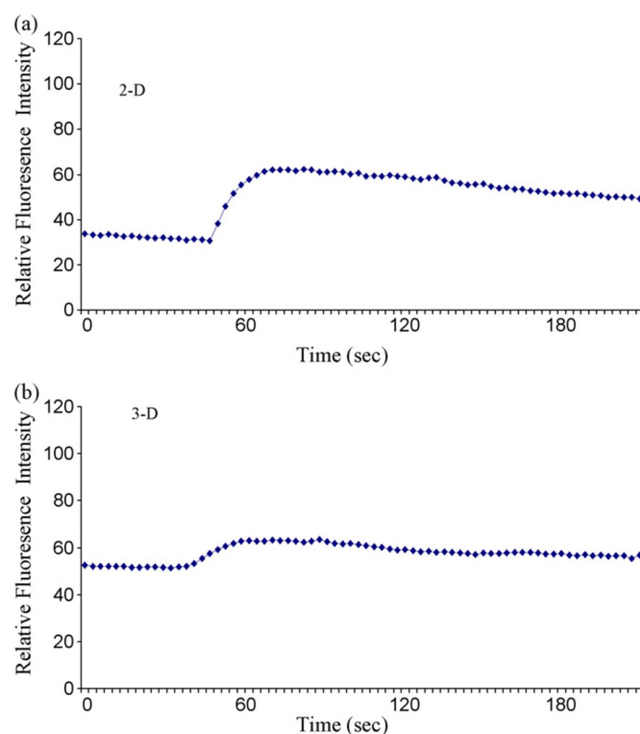

Fig. 5. Typical plots of changes in relative intracellular Calcium Green-1 fluorescence intensities for cells on 2-D SU-8 substrate (a) and 3-D microwell (b) at day 8 into differentiation. The cells in (a) and (b) are from the data pool of 67 and 23 cells, respectively (Table 1). Cells were stimulated by high K^+ depolarization with HBS containing 50 mM K^+. Qualitatively, cells on 2-D SU-8 substrates had a higher response magnitude than cells in 3-D SU-8 microwells. Baseline drift is an indication that cells were not at "steady state", which is common.

Table 1
Percentage of responsive cells and magnitude of VGCC responsiveness to 50 mM K^+ stimulation in dcAMP and BrdU differentiated SH-SY5Y cells.

	3-D SU-8		2-D SU-8	
	Microwells coated with poly-L-lysine		Substrates coated with poly-L-lysine	
	Percentage	Magnitude (mean ± S.D.)	Percentage	Magnitude (mean ± S.D.)
Day 2	14/40 = 35%	0.32 ± 0.11 ($n = 14$)*	67/67 = 100%	1.40 ± 0.50 ($n = 67$)
Day 8	9/29 = 31%	0.24 ± 0.06 ($n = 9$)*,#	23/23 = 100%	1.01 ± 0.31 ($n = 23$)#

A cell was only considered responsive when it showed an increase in Calcium Green-1 fluorescent intensity of 15% or higher over the basal fluorescent intensity level. The percentage is expressed as: (number of responsive cells)/(total number of cells measured) = percentage of responsive cells.

* Values that are significantly different from those for cells on 2-D SU-8 substrates ($p < 0.05$).

\# Values that are significantly different from those for cells on day 2 into differentiation ($p < 0.05$).

cells in microwells, the response magnitude was approximately 1/3. In addition, a similar decrease of the response magnitudes was observed from day 2 to day 8 into differentiation for cells on 2-D SU-8 substrates as well as in microwells ($p < 0.05$).

These results are at odds with the suggestion that microwells are merely "folded" 2-D structure and are not likely to affect cellular function. So this study provides evidence that VGCC function of cells in SU-8 microwells was indeed different from that of cells on planar SU-8 surfaces, which suggest that SU-8 microstructure did affect SH-SY5Y cell differentiation with respect to VGCC function. It has been speculated that cellular function in 2-D is probably an exaggeration of that in 3-D and probably in vivo situation [34]. Results in the present study and previous studies in our lab [8,9,31] are consistent with this speculation. In a previous study with SH-SY5Y cells in collagen hydrogels and Cytodex microbead arrays, the magnitudes of VGCC response of differentiated cells on 2-D substrates were higher than those of cells in 3-D scaffolds. Thus this work is supportive of SU-8 micropattern as a viable platform for engineering quasi-3-D cell culture systems.

The differences between the VGCC function of cells in 3-D pattern and on 2-D surfaces may be due to the cell shape change which is introduced through microstructure. Though, the mechanism by which cells transduce changes in cell geometry into different biochemical responses remains unclear, it is known that cell morphology affects cell growth, cell differentiation as well as cell function and cell death [35–38]. Lascola and Kraig [35] observed cell morphology dependent whole-cell chloride currents in rat astrocytes. To control the cell shape, Parker et al. [37] and McBeath et al. [38] cultured cells on extracellular matrix coated adhesive islands of defined shape and size at the micrometer scale. However, these relatively simple geometric systems only provided a 2-D surface for controlling cell shape and imposed only limited strain on cell shape and dimension. To probe the extent of shape control, it is necessary to challenge cells with a more demanding geometry. Circular 3-D micropatterns fabricated in polymethylmethacrylate were applied to study fibroblasts growth and showed that the patterns in cellular dimention effectively retard fibroblasts invasion [39]. A packed Cytodex 3 microbead array was fabricated for SH-SY5Y cells [31]. In these studies, not only cell shape change was observed, but also cell function change was observed.

Even a neural network-like patterning was achieved by integrating SH-SY5Y cells in the SU-8 microwells with microchannels, SH-SY5Y cells do not form synaptic connection, which suggests that more appropriate candidate cells are needed. In follow-up studies, we will extend our investigation to neural stem cells to engineer 3-D neural networks.

4. Conclusion

Microwell structures were fabricated using SU-8 photoresist for cell-based biosensing application. The microfabrication technique has been well developed and the microstructures' architecture can be well-controlled. The SU-8 platform is transparent and stable, which enables it to be well integrated with optical, biochemical and electrical measurement techniques. SH-SY5Y human neuroblastoma cells were successfully integrated into SU-8 microwells and VGCC function of SH-SY5Y cells on 2-D substrates and in microwells were evaluated. The responsiveness to high K^+ depolarization for cells on quasi-3-D scaffolds was significantly lower than that on 2-D substrates. This result shows that SU-8 microstructure did affect SH-SY5Y cell differentiation with respect to VGCC function and also shows that high-aspect-ratio microstructure is not merely "folded" 2-D structure. This is in agreement with previous 2-D/3-D comparative studies carried out in polymer scaffolds, and supports the speculation that 2-D cell functions may represent an exaggeration of those in vivo. Overall, this work demonstrates the feasibility of using SU-8 microfabricated pattern as a platform for the development of quasi-3-D cell-based biosensors.

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