THREE-DIMENSIONAL TOWER STRUCTURES WITH INTEGRATED CROSS-CONNECTS FOR 3-D CULTURING OF NEURONS

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ABSTRACT

As the complexity of bioengineered systems continues to increase, there is a need for the creation of geometrically-controlled three-dimensional scaffolds for cellular growth and culturing. Since the size scale of micromachined structures meshes well with that of cells, MEMS technology can be utilized for this application. In this work, three-dimensional (3-D) scaffolds have been generated for 3-D cell culturing. The scaffolds consist of high-aspect-ratio SU-8 towers (for high surface area) and towers with three-dimensional cross connect 'bridges', extending from tower to tower, which form highly complex structures for scaffolds. Towers and cross-connects of several dimensions have been tested to find optimal structures of scaffolds for cell culturing. Typical sizes for towers range from 500~700 µm in height and 50~250 µm in diameter. To further promote 3-D culturing, 20~100 µm width cross-connects, bridging from tower to tower, were fabricated. A micro-assembly technique was developed to create a highly complex set of scaffolds from a single tower and cross-connect array. Hippocampal neurons of rat embryos have been cultured on the various developed structures and 3-D interconnection between neurons was successfully observed.

INTRODUCTION

The *in vitro* culturing of living cells is a valuable technique for examining the properties and functionality of different cell types without the limitations of, or reliance on, a living host organism. In addition to the study of cells and cellular networks, cell cultures are an important intermediate step towards the engineering of living tissues, e.g., for prosthetics or other applications. As an example, consider the culturing of neural cells. Two-dimensional neuronal culturing has become routine for basic science studies ranging from toxicity to cell attachment and growth [1][2]. However, it is critical to develop cellular systems for *in vitro* measurement that mimic the 3-D *in vivo* environment as closely as possible, for enhanced cellular survival as well as functionality in networks more representative of tissue-engineered structures and systems [3][4].

As a simple example of a scaffold for growth of 3-D cellular networks, consider an array of high aspect ratio polymeric towers extending normally from an integral polymeric substrate. This configuration facilitates neuronal cell growth on and between the

towers in addition to enabling future perfusion, stimulation and monitoring capabilities via pores and electrodes. 3-D cultures may be expected to be more active in modeling the brain because of greater opportunities for connections in the additional dimension.

Photodefinable epoxies such as SU-8 have been demonstrated to be biocompatible under appropriate conditions [5] and are used commercially in two-dimensional microelectrode arrays for neuronal culturing [6]. Recently, there have been large advances in the fabrication of three-dimensional structures based on photosensitive epoxies [7]. The purpose of this work is to investigate whether such 3-D structures would be suitable for neuronal culturing, and to initiate geometric optimization as a first step toward fully exploiting these 3-D structures.

DESIGN AND FABRICATION

Two types of structures considered as scaffolds for growth of 3-D cellular networks are shown in Figure 1. The first is a relatively simple 'tower' structure, and is composed of high-aspect-ratio SU-8 pillars extending from either glass or silicon substrates. The second is similar, with the exception that multiple-level 'bridges' or cross-connects are suspended from tower to tower to provide additional horizontal surfaces for cells to adhere.

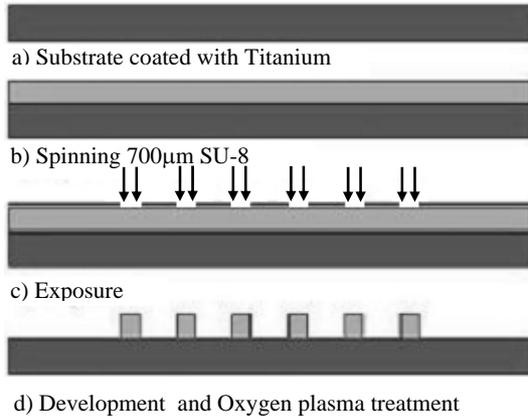The fabrication sequence for the simple 'tower' structure is
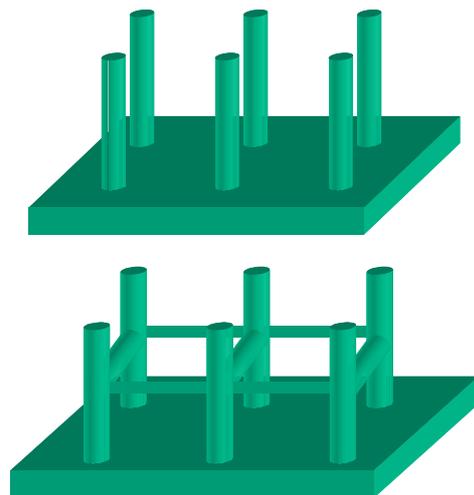
Figure 1. Three-dimensional scaffolds developed in this work. Top: tower structures; bottom: tower structures with hotizontal cross-connects. For clarity, only one horizontal level of cross-connects is shown.
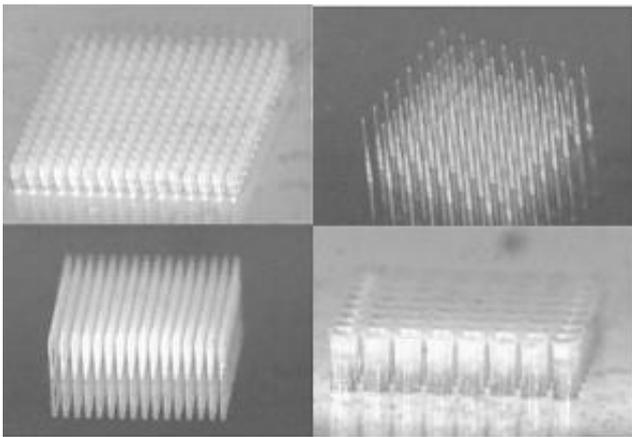
straightforward and is shown in Figure 2. To promote adhesion, RIE treatment is used to roughen the substrate surface. This treatment is followed by the deposition of a titanium adhesion layer. A 700 μm thick layer of SU-8 is then applied and patterned to make the tower structures. The tower diameters and spacing ranged from 50μm to 250μm. Structural tower arrays of 8x8 and 16x16 towers were fabricated and tested as a foundation for cellular culturing of neurons. Figure 3 shows the completed tower structures

a) Substrate coated with Titanium

b) Spinning 700μm SU-8

c) Exposure

d) Development and Oxygen plasma treatment

Figure 2. Fabrication sequence for tower arrays


Figure 3. 16x16 and 8x8 arrays of SU-8 Towers

The fabrication sequence for the cross-connect scaffolds cosists of two parts: generating individual arrays of structures, and assembling them into a complex 3-D structure. Figure 4 shows the fabrication sequence of the individual structures. A 20 μm thick layer of SU-8 is spun on a glass substrate and the tower and associated SU-8 substrate support patterns are subsequently exposed. After post-baking to cross-link the exposed area, an additional 300μm thick SU-8 layer is added without developing the first layer. This additional layer serves as a basis for the cross-connect pattern and extends the substrate. The cross-connect and extended substrate patterns are exposed and post-baked to cross-link the patterns. Finally, all layers of the sample are developed simultaneously.

The individual arrays are then assembled into micromachined molds to form the final scaffold structures as discussed below.
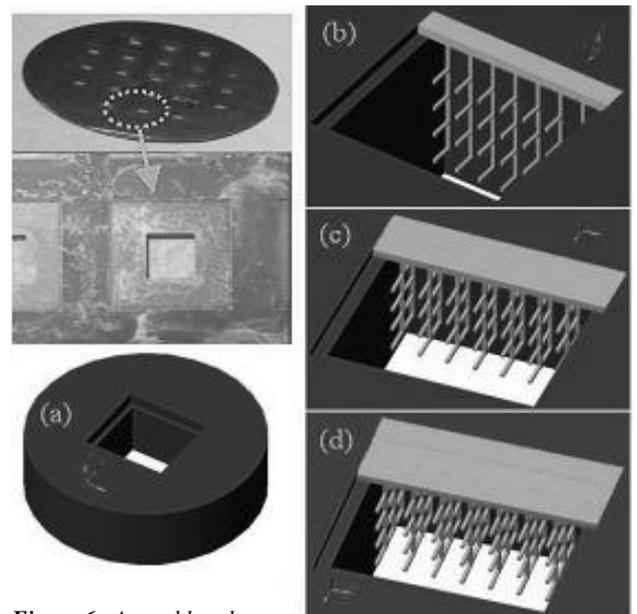
Individual structures with cross-connects are separated from the substrates as shown in Figure 5. Note that each structure consists
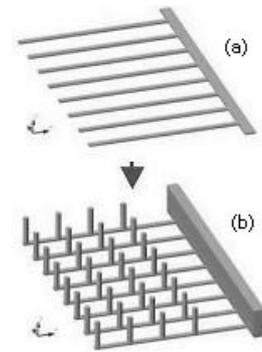

Figure 4. Fabrication scheme of cross-connects
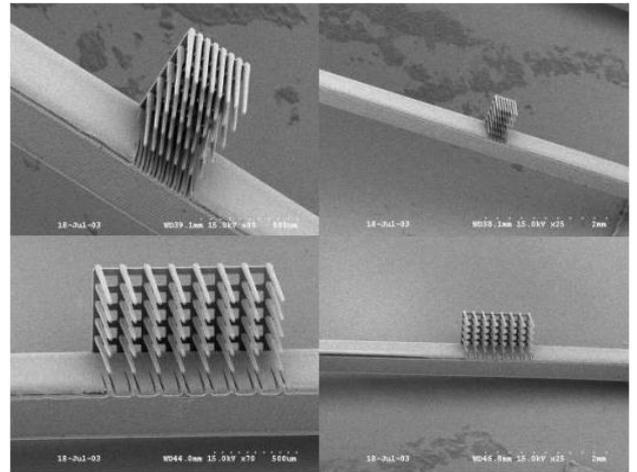
Figure 5. Individual structures with cross-connects

Figure 6. Assembly scheme

of the scaffold structure as well as a substrate 'bar' of material that can be used for handling and assembly. These individual structures are then consecutively placed in an ICP-etched silicon mold as seen in Figure 6. The silicon mold is created by 300 μm deep ICP etching followed by IR-laser cutting to make the square through holes. The 300 μm depth recess is used as a guide to efficiently place the individual arrays. After filling the mold with individual structures, a thin bonding layer of SU-8 is spun on and patterned in squares to adhere the assembled structure and complete the device. To augment biocompatibility, a thin layer of parylene is then coated over the entire surface. Figure 7 shows the completed tower structures with associated cross-connects.
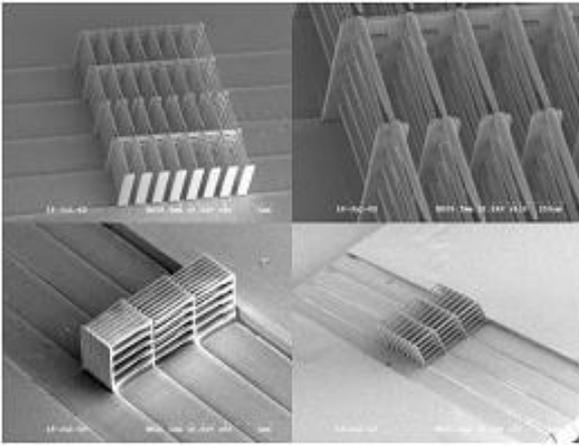
Figure 7. *Completed tower and cross-connect* structures

CELL CULTURE

Prior to application of cells onto the tower structures, a sequence of sterilization steps is required to ensure the viability of the neural culture. First, substrates are soaked in ethanol overnight to leach out any potential organic contaminants that can be toxic to cell growth. After soaking, the samples were dried under vacuum at 90°C for at least 2 hr to remove any solvents that might remain from the soaking process. The samples are then soaked in a solution of PDL (poly-D-lysine, 0.1 mg/mL in deionized water, Sigma) for 1 – 2 hours. PDL is a common protein that promotes cell adhesion. After PDL adsorption, samples are rinsed thoroughly in deionized water to remove any unbound PDL and gently dried with nitrogen gas. Extra care was taken during the blow-dry process so as not to damage the three-dimensional structures. As a final step, the samples were soaked briefly (20 s) in 70 % ethanol to prevent microbial contamination and after blow-drying the sample, it was quickly transferred to a sterile Petri-dish.

Hippocampal neurons are taken from embryonic day 18 rats, mechanically dissociated and delivered in Hibernate E from BrainBits[TM] (Brainbits, Springfield, IL). Hippocampus tissues were triturated and plated at a density of 200 cells/mm^2 under serum-free and glia-free conditions [6]. The cell plating medium was Neurobasal/B27 (Invitrogen, Carlsbad, CA) containing 25 M glutamate and 0.5 mM glutamine. Plated cells were incubated at 37 C in 9% O_2 5% CO_2 through the culture. After 4 days, one-half of the medium was changed with Neurobasal/B27 medium containing 0.5 mM glutamine.

RESULTS AND DISCUSSION

Some preparation steps were required to take pictures of cultured neurons using the scanning electron microscope (SEM). First, half of the media was removed and the culture was rinsed in 1x PBS (phosphate buffer solution, pH 7.4) twice very gently. Fixative, 4% paraformaldeyde in PBS, was added at room temperature for 20 min. After the primary fixation, the culture was rinsed in a buffer (1xPBS) and dehydrated through a series of ethanol solutions in ascending percentages (25 %, 50 %, 75 %, 100 %). After ethanol dehydration, the culture was dried by soaking in hexamethyldisilazane (HMDS) for 3 min twice and put in a dessicator under house vacuum for at least 1 hr. Finally, it was mounted on an SEM stub with carbon stub adhesive and sputter-coated with gold/palladium for 60 sec. After conductive coating, silver paint was utilized to form a ground connection with the conductive coating.

Figure 8 shows SEM pictures of 3-D neuronal cell cultures on SU-8 towers without cross-connects. Cell bridging and web-like connections of 3-D neuronal networks show the possibility of 3-D cell culturing with these scaffolds.
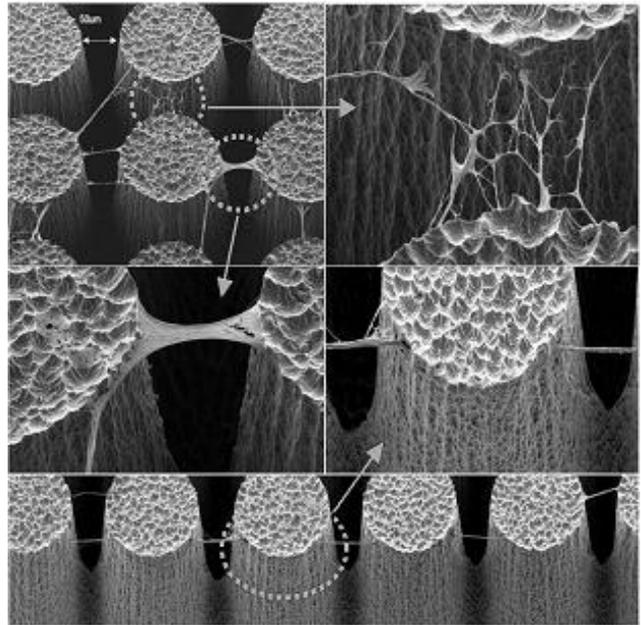
Figure 8. *Cell bridging in 3-D networks of cultured cells*

Figure 9(a) shows a perspective view of 3-D structures with neurons growing on cross-connects. Two neurons which are well spread and attached to the round-shaped surface can be seen in the inset. The outgrowth of neurites are very healthy and presumably making connections between each other. Due to the low plating density, there are a few cells available for each bars. Some cells are bridging the edge and make web-like connections as shown in Figure 9(b). At the left top corner, there is a healthy neuron attached to the vertical wall. One can see growth cones in this cell. Figure 9(c) shows a healthy neuron growth on the flat SU-8 surface beside the 3D structure. The status of the culture was judged by observing the cell growth in this region. Figure 9(a) and

9(b) were taken by SEM while Figure 9(c), 9(d), and 9(e) were made by transmitted light microscopy (phase contrast).
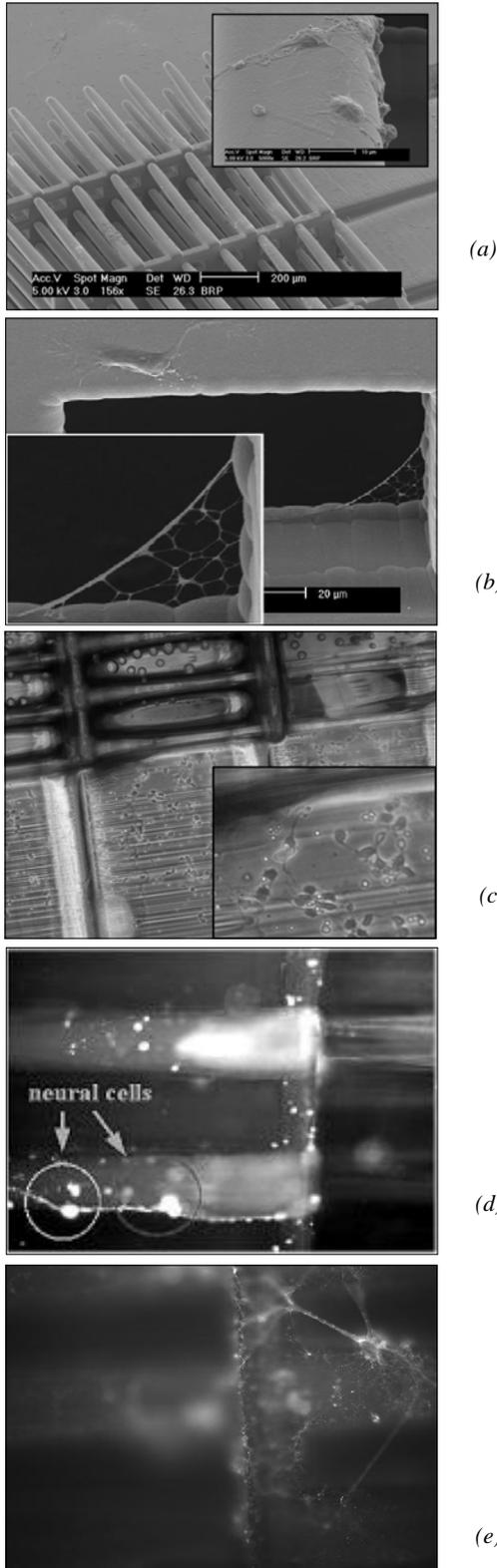

(a)

(b)

(c)

(d)

(e)

Figure 9. *Cell culture on cross-connect*

CONCLUSIONS

This work has demonstrated that cell culturing on complex 3-D MEMS structures, including bridging, is feasible, and opens the possibility of cell growth on highly functionalized scaffolds for tissue culturing and engineering applications. The cross-connect configuration facilitates neuronal cell growth on and between the towers in addition to enabling future perfusion, stimulation and monitoring capabilities via pores and electrodes.

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