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Neuroblasts Micropatterning on Nanostructural Modified Chitosan Membranes

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***Abstract*—**The study describes chitosan membrane platform modified with nanostructure pattern which using nanotechnology to fabricate. The cell-substrate interaction between neuro-2a neuroblasts cell lines and chitosan membrane (flat, nanostructure and nanostructure pattern types) was investigated. The adhered morphology of neuro-2a cells depends on the topography of chitosan surface. We have found that neuro-2a showed different morphogenesis when cells adhered on flat and nanostructure chitosan membrane. The cell projected area of neuro-2a on flat chitosan membrane is larger than on nanostructure chitosan membrane. In addition, neuro-2a cells preferred to adhere on flat chitosan surface region than on nanostructure chitosan membrane to immobilize and differentiation. The experiment suggests surface topography can be used as a critical mechanism to isolate group of neuro-2a to a particular rectangle area on chitosan membrane. Our finding will provide a platform to take patch clamp to record electrophysiological behavior about neurons in vitro in the future.

***Keywords*—**Chitosan membrane, neuro-2a, wet chemical etching, solvent casting.

I. Introduction

ONE of the most significant current issues in biomedical engineering is neuron patterning on artificial scaffolds. The study of neuron behavior on artificial scaffolds that can mimic physiological topography and chemistry surface is becoming a fundamental issue to better understand neural development mechanisms. The mechanisms of neural development are critical issues in biomedical engineering. Numerous studies have attempted to explain neuroscience on artificial scaffolds. For example, Albert Folch et al, presented an in vitro study to investigate how axons of murine embryonic cortical compete growth options on 3-D PDMS substrates [1].

Traditional neural cell culture is composed of random arrangement of neurons and glial cells surrounded by culture medium and culture materials. This is difficult to study neuroscience in vitro because neurons prefer to aggregate into uniform groups. Therefore, scientists use neuron patterning to better understand neuroscience through two approaches. One is surface topographical patterning (physical cues), another one is patterning of regions with various chemicals (chemical cues). Craighead et al. investigated that the different chemical and topographical patterning on silicon substrates would influence the attachment and growth of mammalian central nervous system cells [2]. They used lithography and microcontact printing (μCP) to pattern surfaces with self-assembled monolayers and proteins. The result showed chemical patterns can localize and guide the growth astrocyte glial cells on the silicon surfaces.

In addition, the topography of surface is a critical factor for nerve cells culture. In the nerve tissues, the adhesion and attachment of neuroblasts are mainly mediated by the surface molecular interaction, while the cell morphology and orientation are significantly affected by topography of the matrix. The topography of the extracellular matrix (ECM) plays vital roles in cellular behavior such as adhesion, spreading, migration, proliferation and differentiation [3]-[6]. These experiments suggested that surface texture at the scale of tens of nanometers to micrometers can influence the attachment of the cells to a surface. Therefore, surface topography can be used as a mechanism of isolating cells to a particular area on a silicon substrate.

However, the main drawback of the Craighead， s studies is the material they used. The silicon is an inorganic device and is not appropriate for the biocompatible usages. Moreover, silicon substrate is hard to take real-time observation by inverted optical microscope and must use other treatments, such as SEM or immunofluorescence staining, which increases inconvenience of experiments. Therefore, a novel biocompatible platform was developed in this study to investigate neuroscience by pattern neuroblasts.

In this study, we use chitosan which is a cationic natural biopolymer produced by alkaline N-deacetylation of chitin, the most abundant natural polymer after cellulose. Previous studies showed that fetal mouse cerebral cortex cells can grow well on chitosan film [7]. It becomes an interesting biomaterial due to its good biocompatibility, biodegradability, low toxicity and low cost. Furthermore, in our study, we can pattern neuroblasts on chitosan membrane by using modify chitosan membrane with nanostructure pattern made via photolithography, chemical etching and solvent casting. Nanostructure on chitosan membrane not only can mimic physiological environments in vivo but also can isolate neuroblasts to a specific area on chitosan membrane.

In this study, we analyzed the differentiation dynamics of

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neuro-2a neuroblasts on nanostructure pattern chitosan membrane and observed at 6 hours to 96 hours durations by optical microscope. The study tries to create a chitosan-based neuron patterning platform to get more insight into neurobiology, such as neuron-neuron interaction, neuron-glial interaction, neurite growth, and synaptogenesis in vitro. The future work of this study is to establish neuron network so that scientist can take patch clamp for testing electrophysiological behavior or do neurotransmitter test for creating new drugs.

II. Materials and Methods

A. Fabrication of Nanostructure Pattern Chitosan Membrane

The medium molecular weight chitosan powders (average molecular weight: 190-310 kDa) from Sigma–Aldrich with deacetylation degree about 85% were used. For medium molecular weight chitosan solution, we prepared 1% w/v chitosan solutions by dissolving chitosan powders in 1% v/v of acetic acid. Then these solutions were continuously stirred with a sterile magnetic bar for 12 hours until chitosan powders dissolving in 1% v/v of acetic acid completely. Removal of any undissolved impurities and foam was accomplished using vacuum filtration through a filter paper. Dissolved chitosan solutions were poured into petri dish and dried in an oven at 60°C about 8 hours until the total evaporation of the solvent. Then the substrates were immersed in a 5% sodium hydroxyl solution bath about 6 hours. Theses pristine chitosan substrates were finally rinsed in deionized water until neutral pH value.

The process of nanofabrication for chitosan membrane with nanostructure pattern surface is showed in Fig. 1. The AZ4620 photoresist is firstly coated on silicon wafer and then patterned as the protective layer by photolithography. Following the established protocol, the patterned wafers are selectively chemical etched by a mixture of hydrogen fluoride (HF, 49% wt), hydrogen peroxide (H2O2, 30% wt), and silver nitrate (AgNO3, 0.01 M) aqueous solution for 1.5 minutes at room temperature. After etching, the silver film wrapped on silicon wafer was removed by diluted H2O2 (30% wt). Following, the photoresists were removed by acetone, rinsed with de-ionized water and dried under a stream of nitrogen. The chitosan solution was prepared by dissolving chitosan powers in acetic acid solution (1% v/v). After casting chitosan solution on wafers, the chitosan substrates were dried, peeled off, and dipped into alkaline solution. Finally, the chitosan substrates were then rinsed in deionized water until neutral pH and dried in ambient air.

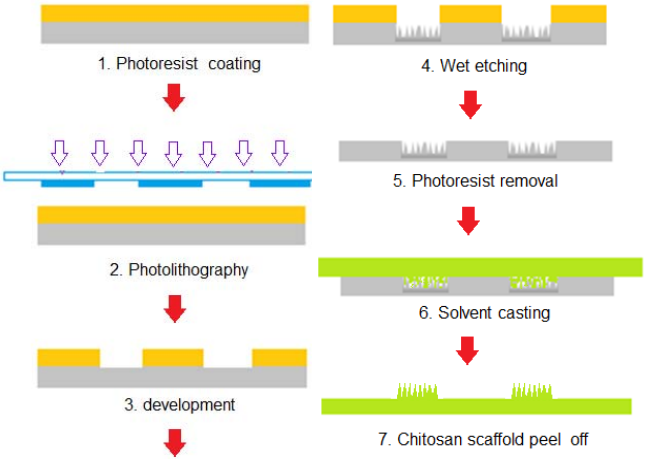

Fig. 1 The fabrication process for chitosan membrane modified with nanostructure pattern

B. Neuroblast Culture

Neuroblasts, neuro-2a cells from American Type Culture Collection (ATCC) were cultured in 90% MEM (Eagle) with Earle's BSS, 2 mM L-glutamine, 1.5 g/L sodium bicarbonate, 0.1 mM non-essential amino acids, 1.0 mM sodium pyruvate and 10% FBS. Before culturing neuro-2a cells on the chitosan membrane, the membrane was first sterilized using UV light and 1% bleaches and then rinsed three times with sterile phosphate-buffered saline (PBS). Neuro-2a cells were washed once with PBS and then exposed to trypsin-ethylenediamine-tetraacetate for 3 min to dissociate them from the tissue culture plates. Next, the cells were seeded onto chitosan membrane and cultured at 37。C and 5% carbon dioxide in growth media supplemented with retinoic acid. Neuro-2a cells were incubated for 6, 12, 24 and 48 hours and then observed cell morphology via an optical microscope.

III. Results and Discussion

A. Surface Characteristics of Chitosan Membrane

After solvent casting process, the chitosan membrane was peeled off from silicon nanostructure made via chemical etching. The top-view and cross-section topography of chitosan membrane were conducted by scanning electron microscope S-4300 (Hitachi, Japan). Before imaging, the chitosan substrates were coated with platinum for 3 minutes by the ion sputter. The surface topography of chitosan membrane employed via scanning electron microscopy was shown in Fig. 2 Fig. 2a shows nanostructure pattern chitosan membrane with flat square region and nanostructure on other regions. The size of chitosan nanostructures were about 400 nm in width and 100 nm in depth. The size of flat square region is 100 µm by 100 µm and the distance between each square is 30 µm. The depth of chitosan nanostructures also can be adjusted through using different molecular weight chitosan powder.

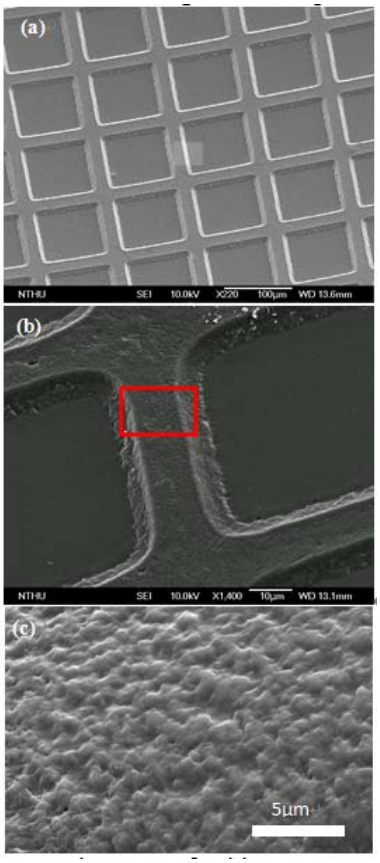

Fig. 2 SEM images of chitosan membranes. (a) Nanostructure pattern chitosan membrane. (b) Surface topography of chitosan membrane. (c) High magnification of surface topography of chitosan nanostructures (red region in (b))

The mechanical properties of chitosan membrane were investigated by AFM in dry and wet conditions as well. The young's modulus of dry and wet chitosan membranes are both about 6 GPa. For most cells, the cell behaviors show different responses while the stiffness of culture substrate is around kPa. Therefore, we can conclude that the neuro-2a behavior would not show distinct difference on chitosan membrane.

B. Neuroblasts Response to Flat and Nanostructure Chitosan Membrane

To probe how neuro-2a cells respond to substrate topography, we examine morphology and cell adhesion area on flat and nanostructure chitosan membrane by optical microscope. Neuro-2a cultured on flat surface showed normal neuroblast morphology and spread to around 250 μm2 in size after 24 hours of culture. In contrast, on nanostructure chitosan membrane, neuro-2a appeared more round-up shape and relatively smaller in size. It suggested that the flat surfaces would provide better cell adhesion than nanostructure surfaces. Fig. 3 shows the projected cell area of neuro-2a on flat and nanostructure chitosan membranes at different cultured durations. The projected cell area was analyzed through ImageJ (public software from National Institutes of Health; http://rsbweb.nih.gov/ij/). Data are mean ± standard deviation (N = 20, n = 4). After 24 hours, the cell area of neuro-2a on flat chitosan membrane will gradually increase and larger than on nanostructure. The result exhibits that the spreading ability of neuro-2a cells could be inhibited by nanostructure.

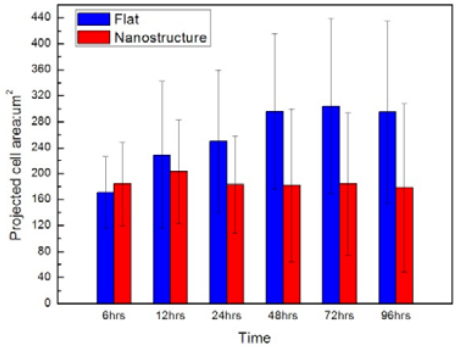

Fig. 3 Projected cell area of neuro-2a after 6, 12, 24, 48, 72 and 96 hours of culturing neuro-2a on flat and nanostructure chitosan membranes. Data are mean ± standard deviation (N = 20, n = 4)

C. Neuro-2a Patterning on Nanostructure Pattern Chitosan Membrane

The neuro-2a cells at a density of 10^5cells/mL were seeded on flat chitosan membrane and tended to aggregate into many uniform groups at about 12 hours (Fig. 4a). The nanostructure pattern chitosan membrane can be used as a platform for investigating neural connection. Fig. 4 (b-d) shows that neuro-2a cells cultured on nanostructure pattern chitosan membrane. The results exhibit neuro-2a cells preferred to attach and grew on the flat region and possibly migrated from nanostructure region to flat square array surface. Neuro-2a cells can be isolated on square region to observe their morphology, neuritis differentiation and cell-cell interaction. Neuro-2a cells would aggregate more if neuro-2a cells proliferated more in square region. The adhesion area of a neuro-2a cell would decrease and the shape would become more round when cell aggregate together at longer culture duration. In addition, the neurites of neuro-2a cells tend to connect with other neurites which differentiation from another neuro-2a on other square regions. From Fig. 4d, we can easily observe neuron network on nanostructure pattern chitosan membrane, which means this novel and biocompatible platform can give us better understand of neurobiology.

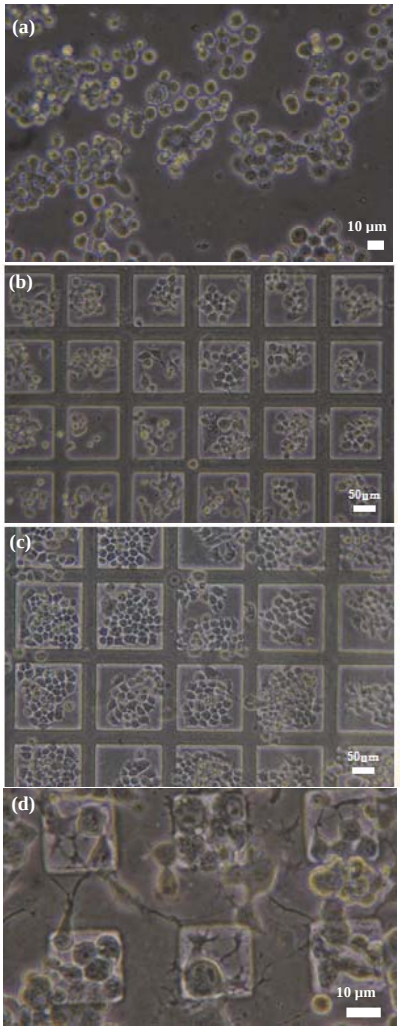

Fig. 4 Neuro-2a cultured on flat chitosan membrane (a) and nanostructure pattern chitosan membrane at 6 hours (b) and 48 hours (c). After 96 hours cultured, neurites of neuro-2a tend to connect with other neurites which differentiation from another neuro-2a on other square region (d)

IV. Conclusion

In this study, we develop an easy-to-handle method which is combined photolithography, chemical etching and solvent casting to fabricate biocompatible nanostructure pattern chitosan membranes to investigate neurobiology. The neuro-2a cells can be patterned to various separate groups according to the surface topography effects. Traditionally, neuroblasts tend to aggregate to form many uniform cell number groups when cultured on flat substrate (e.g., perti dish or flask). It is difficult for scientists to quantitative analyze cell-cell interaction or morphology of neuroblasts. This method is a passive patterning technique which is much easier than using microfluidics to separate neuroblasts and using physical way to pattern nerve cells. This platform can be applied to investigate neuron-neuron interaction, neurite growth and synaptogenesis. In the future, we will work on establishing neuron network so that biologists can do patch clamp to observe electrophysiological behavior and doctor can do drug screening about neurotransmitter test on nanostructure pattern chitosan membrane.

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