Utilizing Nanoscale Patterned Proteins to Create Neuronal Cells Circuits

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ABSTRACT

Micro-patterning of neuronal cells *in vitro* is a critical step for studies in the fundamental biology of neuron-neuron and neuron-surface interactions. The selections of a negative surface modifying agent, such as an extracellular matrix protein like tenascin-C, and a specific positive surface, such as an antibody against neuronal cell adhesion molecules to support cell adhesion on specially designed surface patterns, would be important. The protein modified surfaces could then be used to arrange cells in specific circuits and control cell growth due to a specific protein function such as an inhibition of cell outgrowth. This protein immobilization method on the solid substrates could then be extended to be used as support layers for neuronal cell-based sensors, neuronal networks, biomedical devices, bioprocessing, and bioassays. In this study, we have developed a technique for protein patterning in two dimensions utilizing two proteins for *in vitro* studies of protein patterning to control cell function. Hippocampal neurons were observed to grow axons and dendrites on anti-NGF surfaces. The immobilized tenascin-C surface has demonstrated that tenascin-C inhibits axonal growth while promoting dendrite outgrowth.

Keywords: protein patterning, neuronal cell pattern, SAM, cell-surface interaction

1. INTRODUCTION

Micro-patterning of neurons *in vitro* is a critical step for studies in the understanding of the fundamental biology involved in neuron-neuron and neuron-surface interactions. A couple of investigators have developed patterned substrates to induce formation of neuronal networks from primary neuron culture [1-5]. They have shown that chemically defined substrates can be used to control cell adhesion and neurite outgrowth.

Patterning of surfaces with biological molecules is a method to develop biologically integrated devices with resolutions from micron to the nanometer scale. Protein patterning is currently used for the development of biosensors for cell studies and tissue engineering applications. Several techniques, such as local deposition of molecules using microcontact printing methods, laser techniques, photochemical structuring, and photolithography [6-9] have been examined to generate patterns of functional biomolecules on solid surfaces for applications such as neuronal cell-based biosensors and neuronal networks.

A method of protein immobilization on surfaces for creating protein patterns is physical adsorption where attraction between the solid surface and the protein results in coverage of the surface [10-12]. Another method of protein immobilization is to covalently bind a protein to the surface using a chemical covalent bond [9,13-15]. We are interested in the development and application of techniques for protein patterning in two dimensions with two proteins per surface.

The culturing of patterned neurons on proteins coupled to SAMs requires chemical modification of the SAM surface to induce cell adhesion and to promote neurite outgrowth. It is important to select specific substrates such as extracellular matrix proteins such as tenascin-C, and specific functional proteins, such as anti-nerve growth factor (anti-NGF), that interact with neuronal cell adhesion molecules to support cell adhesion on designed surface patterns. We have examined a technology to create cell patterns based on an initial surface of the 3-mercaptopropyl-trimethoxysilane (MTS) SAM combined with microcontact printing (μCP) of proteins from patterned polydimethylsiloxane (PDMS) stamps. These protein modified surfaces can be applied to create specific cellular patterns and control cell growth because of a specific protein function such as inhibition of cell outgrowth.

In this study we have developed a technique for protein patterning in two dimensions with two proteins on a single surface for *in vitro* studies of protein function by using a combination of the self-assembled monolayer of MTS, a heterobifunctional crosslinker and microcontact printing (μCP) of proteins with patterned PDMS stamps. We have determined that specific regions of proteins (anti-NGF) on the surface can be patterned to attach embryonic day 18 rat hippocampal neuronal cells. The remaining regions are backfilled with a protein, tenascin-C, which has been shown to be anti-adhesive for many cells and to promote cell rounding, but which also has been proposed to mediate neuron glia adhesion and to promote neurite outgrowth. [16-18].

2. MATERIALS AND METHODS

The MTS (Aldrich Chemical Company, Milwaukee, WI) solution was prepared in a glove box. The O_2 level in the glove box was less than 1ppm to prevent oxidation of the SH group of MTS. A volume of 125ml of distilled toluene was transferred to a beaker. Then, 2.5 ml of MTS was added to the toluene by pipette and mixed thoroughly to make a 2% MTS in toluene solution. Prior to coating, the glass coverslips were cleaned in a plasma cleaner (Harrick) at an oxygen pressure of 100 millitorr for 15 minutes. The cleaned glass cover slips were then immersed in the MTS toluene solution for one hour, rinsed in toluene three times, and allowed to air dry.

The N-succinimidyl 4-maleimidobutyrate cross-linker (GMBS) (Fluka) was dissolved in dry DMSO to make a 2mM GMBS in absolute ethanol solution. The MTS-coated cover slips were then immersed in the 2mM GMBS for one hour at room temperature.

After the reaction, the MTS-GMBS cover slips were washed with absolute ethanol three times. After being washed with PBS (1X), the MTS-GMBS cover slips were dried with N_2. Then the Anti-NGF (Chemicon, Temecula, CA) solution was applied to the Polydimethylsiloxane (PDMS) stamp and dried with a flowing stream of N_2. The anti-NGF was stamped on the cover slips for 2-5 minutes. After stamping, the cover slips were incubated at room temperature for more than two hours. Next, the cover slips were immersed in the blocking agent, tenascin-C (Chemicon International, Temecula, CA) solution and were backfilled for one hour. Finally, the cover slips were washed and rinsed with a 0.1% Tween 20 in PBS solution by shaking for 30 minutes, and then washed again with PBS three times. Cell culture was in serum-free media containing neurobasal media, B-27 supplement and glutamine using embryonic day 18 rat hippocampal neuronal cells.

3. RESULTS AND DISCUSSIONS

Figure 1 depicts the reactions during the protein immobilization procedure. The thiol group on the silane reacts with the maleimide region of the heterobifunctional crosslinker, GMBS, by covalent binding for 1 hour. Then the succinimide residues bind terminal amino groups of the antibody, which was applied by microcontact stamping, or of tenascin-C, which was applied by immersion from a buffer solution.

The contact angles of the films were checked as a semi-quantitative guide to evaluate film quality and reproducibility. The cleaned glass cover slip contact angles were less than 5º before reacting with MTS. The contact angle of MTS coated coverslip was 55º+/-3º. The contact angle of the MTS-GMBS coverslips was approximately 45º.

Before evaluating the samples by culturing neuronal cells, it was important to determine the existence of the protein pattern on the glass coverslips. Two glass coverslips were selected as samples for the secondary antibody reactions. Figure 2 indicates that an anti-NGF antibody polarity pattern (backfilling with tenascin-C) that was examined by a secondary antibody reaction using anti-mouse IgG. The picture was taken using a fluorescence microscope.

Anti-NGF molecules were printed on the MTS-GMBS surface using a PDMS stamp, which contained a polarity pattern. The protein tenascin-C was backfilled on the unpatterned area to block the exposed GMBS succinimide residue.

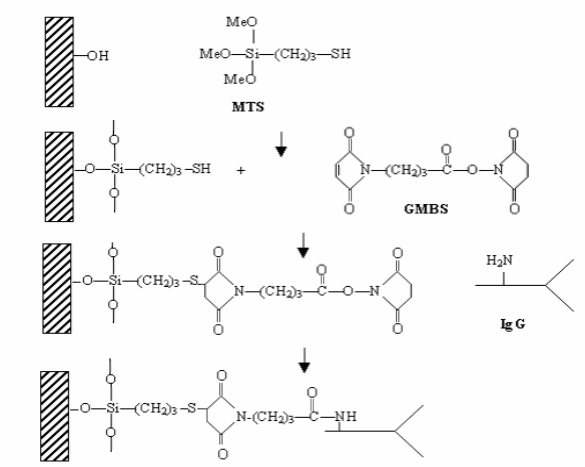

Figure 1: Schematic procedure of protein immobilization

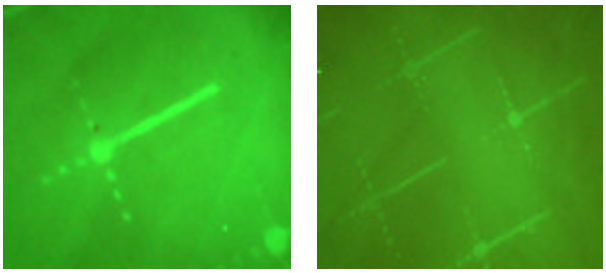

Figure 2: Micrographs of the anti-NGF polarity pattern then backfilled with tenascin-C. The green patterns were used to characterize the patterns by a secondary antibody reaction with anti-mouse IgG. X is the magnification.

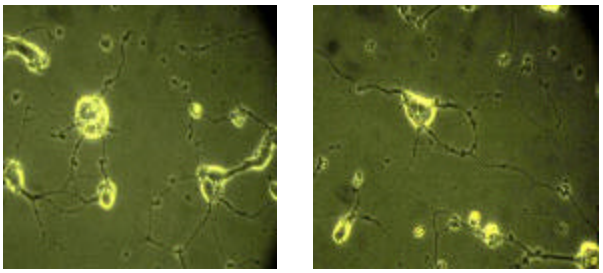

Figure 3: Hippocampal neurons plated on an anti-NGF surface control without patterns. (a) and (b) in day 4 cultures.

Two surfaces were then prepared for comparison of hippocampal neuronal cell growth. Anti-NGF and tenascin-C were immobilized on silanized glass coverslips through the GMBS crosslinker as indicated previously. Figure 3 shows neuronal growth on day 4 on an immobilized anti-NGF surface without patterns. Neuronal cells began to grow and extend processes. Nerve growth factor (NGF) receptor is a polypeptide that has a large effect on survival of peripheral and CNS neurons. In this research, anti-NGF was immobilized by a cross-linking reagent to a glass surface in an attempt to retain its antigen binding ability. Figure 3 shows that the anti-NGF surface promoted the hippocampal neuron attachment and neuronal cell growth on day 4 cultures.

Figure 4 shows that hippocampal neuron growth was limited on a tenascin-C surface on day 4, as only some cells had limited neurite outgrowth. Tenascin is a large glycoprotein that forms disulfide-linked hexamers. It has a multidomain with many repeat structural units including heptad, EFG-like and fibronectin type III repeats, as well as a homology to the globular domain of ß- fibrinogen and ?-fibrinogen. Tenascin is expressed in distinctive spatial and temporal patterns in many embryonic tissues where tissue remodeling and cell migration occur. The tenascin-gene family consists of four distinct genes: Tenascin-C, Tenascin-R, Tenascin-X and Tenascin-Y. Tenascin-C is mainly expressed by glial cells during the development of the central and peripheral nervous system. The biological functions of tenascin are still partly controversial. Research indicates that the extracellular matrix glycoprotein tenascin-C has the function of controlling cell adhesion, neuron migration and neurite outgrowth [16-19].

Tenascin-C has both stimulatory and inhibitory effects on CNS neurons. In some research, tenascin-C was used to study both extension and avoidance behaviors of growing axons and to demonstrate the dual aspects of astroglial effects on developing neuronal tissues [20]. In this experiment, tenascin-C inhibited the hippocampal neurite outgrowth. The reason is not yet clear; however, it may be due to the distinct domains of tenascin-C, which underlies anti-adhesive (against cell-binding) for cells and promotes cell rounding for its inhibitory properties [21].

The results of culturing hippocampal neurons on an Anti-NGF and tenascin-C pattern demonstrated that hippocampal neurons were observed to adhere on the surface of anti-NGF immobilized. Tenascin-C had the ability to adhere hippocampal neurons, but inhibited the neurite outgrowth. Results were observed on day 3 and day 7, which are shown in Figure 5 and Figure 6. Hippocampal neurons grew and made neurites along the pathway of the polarity pattern. The results of the hippocampal neuronal pattern demonstrate that anti-NGF was the dominant factor for cell adhesion and neurite outgrowth on the anti-NGF/ tenascin-C patterns. Even though both tenascin-C and anti-NGF can promote hippocampal neuron adhesion, neuronal cells preferred to grow on the anti-NGF where neurite outgrowth was not inhibited.

By using the competitive properties of different proteins such as extracellular matrix proteins for neuron cell adhesion and growth, further study will focus on selecting and comparing different proteins for microcontact printing and backfilling for an optimal pattern formation of hippocampal neurons. The functionalities of the proteins are also very important after immobilizing on the substrates.

Our further research will focus on keeping the natural functionality of proteins by using several high-affinity ligand pairs to immobilize proteins to substrates such as avidin-biotin, lectins, protein A and protein G, and a fragment of specific antibodies. We believe that the patterns of neuronal cells on the protein patterns are very useful for applications such as neuronal cell circuits and neuronal networks in the near future.

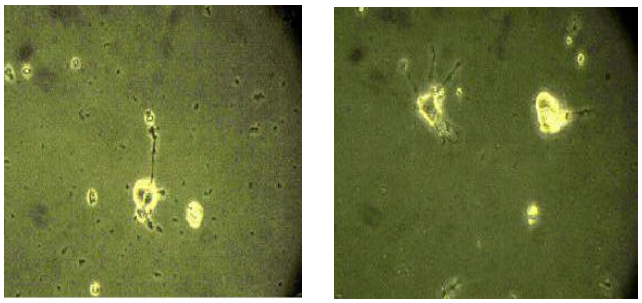

Figure 4: Hippocampal neurons plated on a tenascin-C surface controls without patterns. (a) and (b) in day 4 cultures (morphologically immature processes)

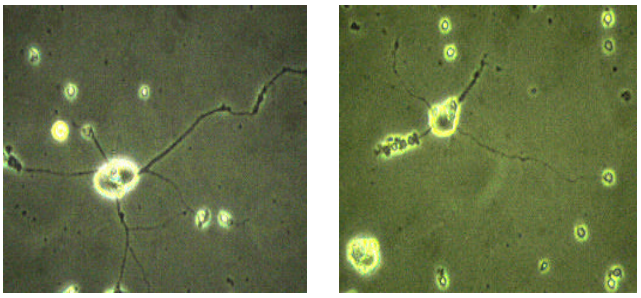

Figure 5: Patterned hippocampal neurons plated on an anti-NGF polarity pattern. The surface was backfilled with tenascin-C. (a) and (b) in day 3 cultures

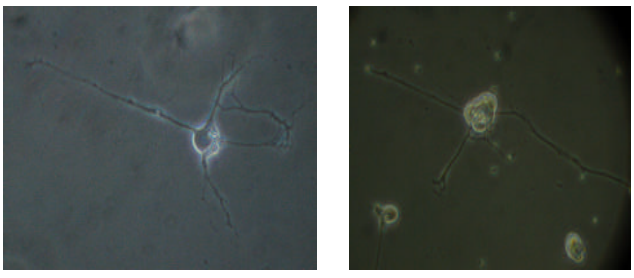

Figure 6: Patterned hippocampal neurons plated on the anti-NGF polarity pattern. The surface was backfilled with tenascin-C. (a) and (b) in day 7 cultures

4. CONCLUSION

Anti-NGF and tenascin-C were immobilized on glass substrates and tested for hippocampal neuronal cell growth. The antibody against NGF was observed to be apositive growth surface while tenascin-C demonstrated that it limited neurite outgrowth. Using the microcontact printing technique, with a subsequent backfilling step, anti-NGF patterns were formed on glass a substrate with a tenascin-C negative region. Patterned hippocampal neurons were achieved on the anti-NGF patterned surface.

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