# CONTROL OF CELL ADHESION AND FUNCTIONS USING SELF-ORGANIZED HONEY COMB-PATTERNED POLYMER FILMS

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Abstract: The design of nano- and microstructures based on self-organization is a key area of research in the search for new biomaterials and biodevices, and such structures have a variety of potential applications in tissue engineering scaffolds and medical implants. 3D scaffolds of appropriate pore size and porosities and with interconnected pores are required to facilitate cell adhesion, proliferation, differentiation, and eventual tissue regeneration in a natural manner. We have reported the honeycomb-patterned polymer film with highly regular pores that is formed by self-organization. The honeycomb films exerted a strong influence on cell morphology, proliferation, cytoskeleton, focal adhesion, and ECM production profiles. Our studies demonstrated that the neural stem / progenitor cells morphology, proliferation, and differentiation are controlled by the pore size of the honeycomb film. It is expected that the honeycomb films will have great potentials as biomaterials for tissue regeneration in a growth factor free proliferation process of stem cells.

## 1 INTRODUCTION

It is well established that surface topography influences implant integration. Many *in vitro* studies have extended these observations to cells in culture, demonstrating that scaffold architecture and surface chemistry considerably influence cell behavior. Therefore, cell adhesion, proliferation, and differentiation can be regulated by controlling surface topography.

We have reported a honeycomb-patterned polymer film (honeycomb film) with regular pores, which is formed by self-organization. The honeycomb films strongly affected cell morphology, proliferation, cytoskeleton, focal adhesion, and extracellular matrix (ECM) production profiles (Tanaka, 2006., Tanaka, 2007., Yamamoto, 2007., Mcmillan, 2007., Tanaka, 2008., Arai, 2008., Tsuruma, 2008.). These studies were performed on cells cultured in the absence of growth factors.

In neural tissue engineering, the preparation of neural stem/progenitor cells (NSCs) is required for the treatment of diseases of the nervous system (Steinman, 2003). NSCs are self-renewing, immature, undifferentiated, and multipotent cells. They can differentiate into cells constituting the central neural system, such as neurons, astrocytes, and oligodendrocytes (Roberti, 2000., Wurmser, 2004., Wang, 2006.). The use of NSCs is a potential therapy for diseases of the nervous system. In order to increase the feasibility of this technique, viable method, the preparation, culture, and seeding of NSCs are steps that have to be carefully controlled. The main problem associated with the use of stem cells is that when these cells are extracted from an



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individual, they start differentiating (specializing into a specific cell type); thus, they lose their stem cell characteristics. Thus, the reintroduction of these cells into patients is problematic. In order to overcome this problem, a culture environment wherein the stem cells can be maintained in the undifferentiated state is required. The proliferation of self-renewing NSCs is required for cell therapy. We studied the effects of the pore size of the honeycomb films on the proliferation and differentiation of NSCs.

## 2 MATERIALS AND METHODS

Honeycomb films were fabricated using biodegradable polymers poly(ε-caprolactone) (PCL) and a copolymer of dodecylacrylamide and ω-carboxyhexylacrylamide. The honeycomb film was prepared on a glass substrate by employing a previously described method (Sato, 2002., Tanaka, 2004., Tanaka, 2007). The flat film was prepared by a spin coater in dry condition. NSCs were derived from the cerebral cortex of embryonic 14 day mice. The NSCs were seeded on the films at a density of $2 \times 10^4$ cells/cm$^2$. NSCs were cultured in serum medium (Opti-MEM, 10 % Fetal Bovine Serum, 55 μM 2-mercaptoethanol) for 24 hr. After that, NSCs were cultured in serum-free medium. The morphologies of neurons were examined by a scanning electron microscope (SEM) and a confocal laser scanning microscope. NSCs were immunostained for Nestin and BrdU. Cell number was estimated by measuring of DNA concentration from the extracted samples.

## 3 RESULTS AND DISCUSSION

Scanning electron microscopy (SEM) revealed a highly regular hexagonal arrangement of pores (honeycomb pattern), and a well-interconnected, uniform pore structure (Fig. 1a–d). NSCs cultured on flat films differentiated into round neurons with neurites that extended randomly on the film. The morphology of cells on the honeycomb films depended on the pore size of the films. On films with a pore size of 1.5 μm, the cell bodies were flat. Their neurites extended randomly and jumped over the pore of the film. On films with a pore size of 10 μm, the cell bodies were round and the cells adhered to the rims of the films. Their neurites extended along the rims forming a simple network. The honeycomb film provided positive cues to support neurite extension. The cells on films with pore sizes of 5 and 8 μm exhibited a morphology similar to that of cells on films with a pore size of 10 μm. Interestingly, on honeycomb films with a pore size of 3 μm, several large spheroids were observed. The neurites gathered to form large bundles, which radiated out from the periphery of the spheroids. NSCs on honeycomb films with a pore size of 3 μm formed spheroids of diameter 30~90 μm (Fig. 2); such spheroid formation was not observed for NSCs either on other honeycomb films (with pore sizes of 1.5, 5, 8, and 10 μm) or on flat films.

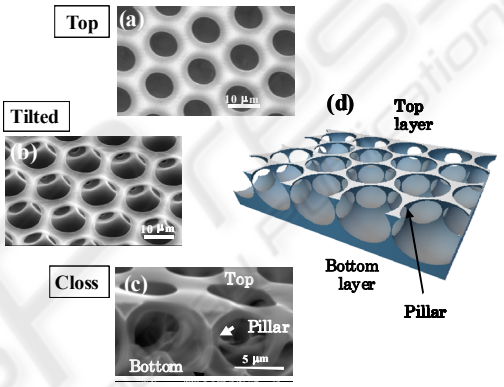


Figure 1: SEM images of the honeycomb film in different views. (a) Surface, (b) tilted, and (c) cross-section. (d) Schematic representation of the 3D structure of the honeycomb film.

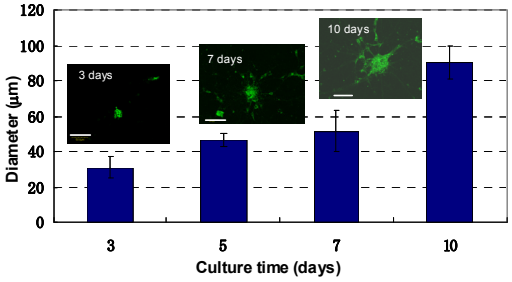


Figure 2: Spheroids diameter on the honeycomb film (pore size:3 μm). Fluorescent images indicate spheroids stained for Nestin. Bar: 50 μm.

In order to characterize the cells on the flat and honeycomb films, the cells were immunostained for nestin and microtubule-associated protein 2 (MAP2) and were labeled with bromodeoxyuridine (BrdU). Nestin and MAP2 are selective markers for NSCs and neurons, respectively. Nestin expression decreases when NSCs differentiate and mature into neurons. BrdU is selectively incorporated into the nuclei of proliferating cells, and thus indicates cell

growth. Immunostaining for nestin and labeling with BrdU revealed that the spheroids were aggregates of self-renewed NSCs. The diameter of the spheroids increased with the culture time (Fig. 2).

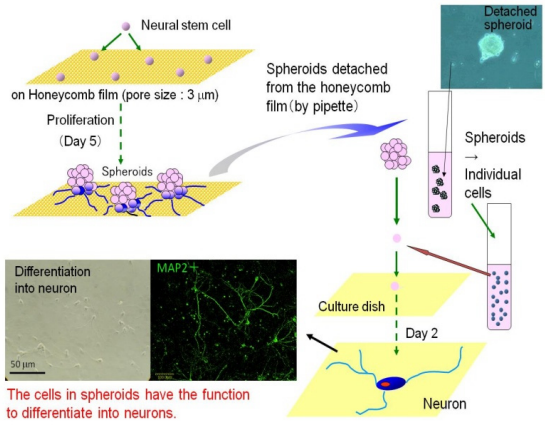


Figure 3: The removability and phenotype of the spheroids on the honeycomb film (pore size: 3 μm).

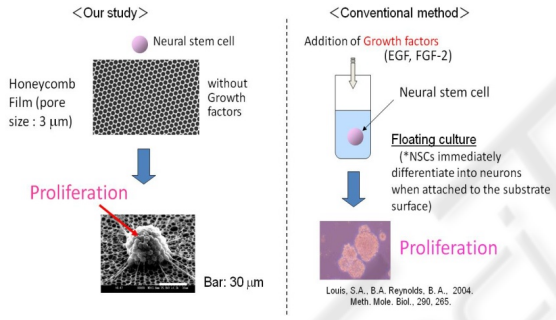


Figure 4: Comparison of our method for proliferation of NSCs using honeycomb films with the conventional neurosphere method.

These results implied that honeycomb films with pore size less than the cell size promoted the proliferation of NSCs, but prevented their differentiation. We found that the number of total neural cells increased after 3 days owing to the maintenance of the undifferentiated state and to the proliferation of NSCs.

In order to determine the removability of the spheroids from honeycomb films and to ascertain the phenotypes of the cells, cells obtained from the spheroids were cultured in a standard culture dish (Fig. 3). All cells of the spheroids adhered to the culture dish. The cells extended neurites after 2 d and were positive for MAP 2, suggesting their differentiation into neurons. This result implied that the cells in the spheroids could differentiate into neurons and confirmed the finding of the immunostaining experiment that the spheroids were aggregates of self-renewed NSCs.

The conventional neurosphere culture method is widely used for the proliferation of NSCs (Cattaneo, 1990., Louis, 2004). This technique involves the use of serum-free culture medium supplemented with growth factors (fibroblast growth factor-2: FGF-2 and/or epidermal growth factor: EGF) (Fig. 4). The NSCs obtained by this method are expected to be supplied to lost and dysfunctional nervous systems in order to regenerate neural tissue. In this technique, NSCs are cultured without the attachment to a surface (floating culture) because the NSCs immediately differentiate into neurons when they are attached to the substrate surface. Improvements in the neurosphere culture technique, which include the use of U-bottomed wells, have recently been reported (Mori, 2006). However, our technique required neither growth factors nor the floating culture system wherein the cells are not attached to a surface.

In our technique, some NSCs were observed to be encapsulated by the 3 μm pores immediately after cell seeding. At this adhesion arraignment, the NSC contacts to the pore at around the cell body. Such circular adhesion may result in a small adhesion area; thus, the NSCs are in an environment similar to that in the neurosphere method, that is, they are suspended to prevent contact with surfaces. Thus, cell encapsulation, in contrast to cell adhesion that is observed on flat surface, is probably the reason for the control of NSC proliferation by surface topography; such encapsulation is characteristic to the honeycomb film with a pore size of 3 μm.

## 4 CONCLUSIONS

Our study revealed that the morphology, proliferation, and differentiation of NSCs are controlled by the pore size of the honeycomb film. This is a novel approach to NSC culture in regenerative medicine, wherein the proliferation and differentiation of the NSCs are controlled by the surface topography of scaffolds. Honeycomb films are potentially useful biomaterials for neural tissue regeneration, which can help in the proliferation of NSCs in the absence of growth factors.

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