Cultivation of Rat Nerve Cells on Nanoimprinted Polydimethylsiloxane Culture Sheets

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Regenerative medical research has attracted significant attention in the medical field in recent years. Here, we fabricated micro trench structures with varying widths of 15 and 150 μm and depths of 30 and 60 μm using the nanoimprint technique on a 100 μm thick polydimethylsiloxane culture sheet used as a biocompatible polymer culture sheet. Rat neurons were observed to grow after 3 days selectively within those micro trenches with widths larger than 15 μm, and they were coated with polylysine. Accumulation of glial cells was also observed in the trenches.

Keywords: rat neurons, culture sheet, nanoimprinting

1. Introduction

Regenerative medical research has attracted remarkable attention after the development of induced pluripotent stem (iPS) cells [1]. Current focus in this field of research aims at restoring the function of the nervous system after trauma. The transplantation of neural stem cell suspension into an injured area has proven to be an effective treatment method. Several reports related to possible treatments of extensively injured areas and extension of remedy time have been published [2-10]. However, the shortcoming of this research is mainly associated with the limited ability of the nerve cells to regenerate. Other interesting areas of regenerative medicine are related to the artificial fabrication of the lost human functions such as the nervous system in a laboratory setting [11-18].

Polydimethylsiloxane (PDMS) films are very flexible and stretchable films and various kinds of micro patterns have been fabricated onto these using microelectromechanical system (MEMS) techniques such as nanoimprinting [12,19-21]. Here we report the cultivation of nerve cells on PDMS culture sheets with micro trench structures on a PDMS substrate fabricated using nanoimprinting.

2. Experimental

2.1. Mold fabrication

Cell culture sheets bearing micro-trenches were fabricated on flexible PDMS sheets using nanoimprint techniques [22,23]. Figure 1 shows the designed mold patterns and eight trenches patterns. Width, W, was adjusted between 15 and 150 μm, length was 9 mm, and the spacing between these patterns was 1 mm.

Fig. 1. Designed mold patterns for eight micro-trenches with width, D, between 15 and 150 μm; length of 9 mm; and spacing of 1 mm.

Figure 2 shows the process of mold fabrication using MEMS microfabrication. Firstly, a mask layer consisting of aluminum (Al) and bearing a thickness of 2 μm was thermally deposited on a 530 μm thick silicon (Si) wafer [Fig. 2 (a)]. A photoresist (S1818, ROHM) was then spin-coated on the Al surface, followed by baking for 20 min at 90 °C [Fig. 2 (b), (c)]. The trench patterns were photolithographically defined using the photomask [Fig. 1 and Fig. 2 (d), (e)]. The trench patterns were developed using a developer (MF319), followed by wet etching of Al with a mixed acid solution and post-baked for 30 min at 120 °C. Following this, Si was etched by inductive plasma...
etcher (MUC21, Sumitomo Precision Products) [Fig. 2 (f)]. The mold was completely formed after the removal of the mask layers [Fig. 2 (g)]. Since two types of trenches possessing different etching depths, $D_s$, of 30 and 60 μm were used in this work, two types of molds were fabricated.

The Si mold was firstly placed on a polyethylene terephthalate (PET) sheet used for overhead projection and 0.3 g of PDMS solution (SILPOT 184 W/C, Dow Corning Toray Co., Ltd) was dropped on the mold [Fig. 3 (a)]. PDMS was then extended into a thin film by placing another PET sheet on the PDMS solution. After confirming that the PDMS solution uniformly covered the entire surface of the mold, the PET sheet was peeled off from the mold surface. The PDMS coated mold was then placed in a vacuum chamber at 0.06 MPa for 15 min for removal of any air bubbles trapped in the PDMS solution [Fig. 3 (c)]. The PDMS layer was solidified in a vacuum oven at 80 °C for 90 min, and PET present on the reverse side of the mold was removed [Fig. 3 (d), (e)]. PDMS adhering to the mold was peeled off by immersing the mold in an ultrasonic bath filled with hexane [Fig. 3 (f)]. Figure 4 (g) shows a photograph of the fabricated PDMS culture sheet. Polylysine $[(C_6H_{12}N_2O)_n]$ was coated on the culture sheet and the surface was wiped using a cotton sheet, following which the culture sheet was dried.

As seen in Fig. 5, polylysine layers were confirmed to remain within the micro trenches. Culture sheets without polylysine coating were prepared and compared to those bearing it in order to investigate the effectiveness of the segregation of nerve cells at the polylysine-filled trenches. The use of polylysine was determined based on previous publications, which demonstrated selective growth of cells on those surfaces of the culture sheets specifically treated with polylysine [20-25, 27].

3. Results and discussions

The nerve cells used in this research [MB-X0505D, rat nerve cells ST(R)] were purchased from DS Pharma BioMedical Inc, Osaka, Japan. All the necessary chemicals such as Hanks solution (HBSS), enzyme liquid, dispersion liquid, and removal liquid were contained in this product. Culture conditions were determined based on previous reports [23-27]. The Hanks solution is a balanced salt solution that provides an environment suitable for the maintenance of structural and physiological integrity of mammalian cells in vitro.

Culture sheets were sterilized by baking at 140 °C for 4 h in a baking oven and the polylysine solution was sterilized by UV irradiation for 10 min. Culture sheets were placed at the bottom of a plastic Petridish, which was filled with the culture solution containing nerve cells. Cells were cultured for 5 days in an incubator at 37 °C and at 5% CO₂ concentration. Cells were taken out from the incubator after 3 days for observation and the cultivation solution was replaced. Following this, the cells were cultured further for 2 days. Nerve cells were observed using an optical microscope after 3 and 5 days of cultivation.

Segregation of the nerve cells within the trenches was observed after 3 days of culturing. Figure 6 (a) - (f) shows the distribution of nerve cells on the culture sheets after 3 days. Although nerve cells were found outside the trench in some cases, most of them appeared to be segregated in the trenches with $W$ between 15 and 150 μm and independent of $D$ on the culture sheets with or without polylysine treatment [Figs 6 (a), (e)]. However, in some cases a higher number of nerve cells were found to spread outside of the trench with $W = 15$ μm as seen in Fig. 6 (f).

Figure 7 shows an example of the surface of the culture sheet after 5 days of cultivation. At this point, most of the nerve cells had disappeared, whereas some glial cells were retained within the micro trenches. Glial cells are non-neuronal cells and function to as glue for connecting the nerve cells in the nervous system.

![Fig. 5. Micro trenches on the PDMS culture sheet filled with polylysine solution.](image)

![Fig. 6. Distribution of nerve cells on the culture sheets after 3 days of culture. (a), $D = 60$ μm, $W = 50$ μm with polylysine treatment; (b) $D = 30$ μm, $W = 80$ μm with polylysine treatment; (c), $D = 30$ μm, $W = 15$ μm without polylysine treatment; (d) $D = 60$ μm, $W = 150$ μm without polylysine treatment; (e) $D = 30$ μm, $W = 15$ μm with polylysine treatment; and (f) $D = 30$ μm, $W = 15$ μm without polylysine treatment.](images)
Unfortunately, most of the nerve cells were rendered useless due to contaminations that were probably introduced during their observation in air, which is consistent with previously reported situations [28-33]. Figure 8 shows the surface of the contaminated culture sheets after 5 days of cultivation. Various kinds of molds were observed on the surface of the culture sheets suggesting the necessity of cleaner or sterile cell cultivation for further studies. Further investigations and optimization are needed to realize the growth of nerve cells into highly organized neuronal systems.

![Nerve cells and Glial cells](image)

**Fig. 7.** Photograph of a culture sheet after 5 days of cell culture

![Contamination](image)

**Fig. 8.** Contamination on the culture sheets after 5 days of cell culture

### 4. Conclusion

We fabricated micro trench structures of varying widths and depths using the nanoimprint technique on a 100 μm thick polydimethylsiloxane culture sheet used as a biocompatible polymer culture sheet. Rat neurons, called corpus striatum, were selectively cultivated after 3 days within the micro trenches filled with polylysine and which had widths larger than 15 μm. Accumulation of glial cells was also observed in the trenches.

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