Growth Behavior of SHSY5Y Cells on Hybrid Micro-pit and Nano-pillar Arrays

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Abstract—The directional arrangement and extension of cells is of great significance in the tissue engineering field. Great efforts have been made to study the effects of micro- or nano-structures on cell behaviors, but the they are still poorly understood. In this work, hybrid micro/nano structures prepared by combining laser marking technology with metal assisted chemical etching (MACE) were introduced to study their effect on the growth behavior of SHSY5Y cells. It was found that the cells on the silicon micro-pit arrays (SiMP array, unetched substrate) were arranged orderly along the edge of the micro-pits, stretched and connected with each other, while the cells on the hybrid silicon micro-pit and silicon nano-pillar arrays (hybrid SiMP/SiNP arrays, etched substrates) were also arranged in an orderly manner with a relatively short cell stretch, but displayed a preference for independent growth. In addition, about 90% of cells showed a preference for growing on the area of nano-pillars (NPs), and only 10% of cells on the area of micro-pits (MPs) on the etched substrate. The results showed that the hybrid SiMP/SiNP arrays trapped cells and restricted the cell spreading. Thus, this approach is of great significance for the study of independent growth behavior of cells on the substrate in the field of single neuron research.

Keywords—silicon nano-pillar, silicon micro-pit, cell behavior

I. INTRODUCTION

Extracellular matrix (ECM), the microenvironment of cells in vivo, has topographic features ranging from the nano- to micro-scale, providing abundant biophysical clues for cells. Studies have shown that the micro/nano topography of ECM, such as pores [1, 2] and fibers [3], is involved in the control of cell behaviors, and their effects on cell activities need to be further revealed. The advance in micro/nano fabrication techniques offers a useful tool for the design of substrate topography for cell research in vitro. In recent decades, various types of materials and topographic patterns have been applied to investigate the interactions of cells with micro/nanopatterned structures, which is of great significance in cell activities, including adhesion [4, 5], migration [6, 7] and proliferation [8, 9].

Among the topographic patterns, pillars and pores have received much attention due to their significant effects on cell adhesion and spreading, which are useful in biomedical engineering, especially in the vascular repair field where the specific adhesion and weak intercellular communication of cells are beneficial [10-12]. For nanopillars, some studies reported that nanopillars enhanced cell adhesion and restrict cell spreading [13-15], which were useful in cell capture for the research of circulating tumor cells (CTCs). Similarly, some methods were developed to study the effect of nanopores on cell behaviors. For example, some approaches reported that the cells cultured on nanopores showed a flattened morphology with the filipodia attached to the pores [16] and developed fewer and shorter neurites compared to the smooth substrates [17]. Pores (or pits) are mainly used in the field of cell localization [18-20]. However, the above micro/nano structures for cell research are usually a single type of structures, which only provide one type of topographical clues, which are far from the complex topographical clues in vivo. Hence, the hybrid structures are an attractive topic, and their effects on cell behaviors need to be further studied.

Based on the above facts, by culturing SHSY5Y cells on SiMP arrays and hybrid SiMP/SiNP arrays, the influence of micro/nano structures on cell behaviors was studied. Here, the SiMP arrays were fabricated by fiber laser marking machine, and then they were processed by etching solution to obtain hybrid SiMP/SiNP arrays (etched substrates). The scanning electron microscope (SEM) was used to observe cell growth behaviors.

II. METHOD AND EXPERIMENT

A. Substrate Preparation

The P(100) silicon wafers (1× 1 cm²) were sequentially cleaned with acetone, ethanol and deionized water under ultrasonic conditions, followed by 10 min of oxygen plasma treatment and 15 nm Ag film deposition. The fiber laser marking machine (FB20-1, Changchun New Industries Optoelectronics Technology, China) with the frequency of 20 kHz and scan speed of 750 mm/s was used to fabricate micro-pit arrays with the period of 100 µm and diameter of 30 µm. After these steps, the silicon wafer with micro-pit arrays was obtained (unetched).
Next, the silicon wafer with micro-pit arrays was immersed into etching solutions (10% HF and 0.6% H₂O₂, v/v) for 45 min, and then treated in HNO₃ (concentration of 65%–68%) for 30 min to remove Ag. Finally, the etched substrate with hybrid micro-pit and nanopillar arrays (hybrid SiMP/SiNP arrays) was obtained. Before the cell experiment, all the substrates (unetched and etched) were soaked with deionized water for 2 h.

B. Cell Culture

SHSY5Y cells (human neuroblastoma cell) were used in this work. 30×10⁴ cells mL⁻¹ were maintained in the DMEM supplemented with 10% FBS and cultured in an incubator at 37°C and 5% CO₂ for 24 h. All samples were treated with the ultraviolet lamp (1 h) and the PBS solution (3 times) before cell seeding.

C. Cell Fixation

The cells were rinsed with PBS, fixed in the 4% paraformaldehyde solution overnight (4°C). After two or three times rinsed again with PBS, the cells were dehydrated in the gradually increased concentration of ethanol solution (50%, 70%, 80%, 90%, 95% and 100%). To further dehydrate the cells, the samples were mixed with tert-butanol overnight (-20°C), and evaporated in the vacuum oven (20 min). At last, a 5 nm of gold film was deposited on the sample surface.

D. Quantification of Cell Behaviors

Image J software was used to analyze the cell length (Feret’s diameter) [21] and the percentage of cells in different areas on the etched substrate. 60 cells were selected from each of 6 samples to measure the length of cells cultured on the unetched and etched substrates. Furthermore, the total number of cells in the region of 0.27 × 0.41 mm² from each of 6 samples was calculated to analyze the percentage of cells in different areas on the etched substrate.

III. RESULTS AND DISCUSSIONS

In this work, the micro-pit arrays were fabricated by fiber laser marking technology, and the hybrid silicon micro-pit and silicon nanopillar (hybrid SiMP/SiNP) arrays were fabricated by further etching. Fig. 1 shows the schematic diagram of fabrication process of the hybrid SiMP/SiNP arrays. The micro-pit substrate prepared by the laser marking machine (Fig. 1 (a)) was then etched in the etching solution for 45 min (Fig. 1 (b)), followed by 65%–68% HNO₃ treatment for 30 min (Fig. 1 (c)). After these steps, the hybrid SiMP/SiNP arrays were formed, as shown in Fig. 1 (d). Note that the treatment by HNO₃ is to eliminate the effect of Ag element on the cell experiment.

Fig. 2 shows the SEM images of the micro-pit array substrate before and after etching. Fig. 2 (a) presents the micro-pit arrays fabricated on the surface of the 15 nm Ag film coated silicon wafer by fiber laser marking machine. The single micro-pit with the diameter of 30 μm is clearly shown in Fig. 2 (b). It has also been noticed that there are some Ag nanoparticles around the micro-pits. This is due to the high temperature generated by laser ablation that dissolves the Ag film, and the dewetting tropism makes it recombine to form Ag nanoparticles, which are marked with the yellow dashed lines as shown in Fig. 2 (b). After 45 min etching and 30 min HNO₃ treatment, the hybrid SiMP/SiNP arrays are formed and shown in Fig. 2 (c). Fig. 2 (d) further shows the details of Fig. 2 (c).

It can be clearly seen that after etching, the area where the silver film exists eventually forms Si nanopillars (SiNPs), and the area where the Ag nanoparticles exist forms Si nanoholes (SiNHs). The formations of hybrid SiMP/SiNP arrays involve the metal assisted chemical etching (MACE) [22-25] and silver plays a catalytic role in this process. Specifically, an oxidation-reduction reaction occurs at the Ag/Si interface, causing Si to continuously etch downward, forming SiNPs or SiNHs. However, there is no silver exist in the micro-pits, so there is no change in the micro-pits after etching.

Fig. 3 shows the growth behavior of SHSY5Y cells on the SiMP array substrate (unetched) and hybrid SiMP/SiNP array substrate (etched). The cells on the SiMP arrays are arranged orderly along the edges of the micro-pits and connected with each other (Fig. 3 (a)), while the cells on the hybrid arrays are
also arranged orderly but displayed a preference for independent growth (Fig. 3 (b)). Figs. 3 (c) and 3 (d) further show the details of the cells cultured on the two substrates. Specifically, on the SiMP arrays, the cells close to the edges of the micro-pits and the lamellipodia are clearly observed (Fig. 3 (c)). The results suggest that the edges of the micro-pits play a topographical guiding role, making the cells grow in an orderly arrangement along the edges of the pits [26-28]. While on the hybrid arrays, the cells are not close to the edges of the micro-pits but on the nearby nanopillars, and no lamellipodia are observed (Fig. 3 (d)). Studies have shown that nanopillars play a role in cell capture by limiting cell spreading and migration [29-31]. Therefore, under the dual effects of topographic guidance of the micro-pits and cell capture of the nanopillars, the cells still grow in an orderly manner, but they are no longer close to the edges of the micro-pits, and show a preference for growing on the nanopillars.

Fig. 3. SEM images of SHSY5Y cells cultured on the SiMP array substrate (a) and hybrid SiMP/SiNP array substrate (b). Scale bar: 50 μm. (c) and (d) show the SEM images of (a) and (b) at high magnification, respectively. Scale bar: 20 μm.

The morphologies of cells on the two substrates from a qualitative perspective are shown in Fig. 3. In order to study the characteristics of cells more comprehensively, the statistical analysis of cell length is shown in Fig. 4. The average cell length on the SiMP arrays (unetched, 117 μm) is longer than that on the hybrid SiMP/SiNP arrays (etched, 74 μm). Since the cells tend to grow in the area of nanopillars on the etched substrate as shown in Fig. 3, and the nanopillars as mentioned above restrict cell spreading, so a large number of cells on the etched substrate with the restricted spreading growth. Therefore, the cells on the etched substrate have a smaller cell length compared to that on the unetched substrate.

It is clearly presented in Fig. 3 that the cells show a preference for growing on the nanopillar area on the hybrid arrays. In order to visualize the phenomena intuitively, the percentages of cells on the nanopillars (NPs) and micro-pits (MPs) located on the hybrid arrays (etched substrate) are quantified, as shown in Fig. 5.

It can be seen that, about 90% of cells on the area of NPs, but only 10% of cells on the area of MPs. The results suggest that the NPs on the hybrid arrays (etched substrate) still have a trapping effect on cells. Therefore, the growth behavior of cells on the hybrid SiMP/SiNP arrays can be concluded as: the topographic guidance provided by the edges of the micro-pits makes the cells orderly arranged. In addition, the nanopillars trap the cells by restricting the cell movement during the cell migration process, so the cells cannot reach the edges of the micro-pits. Finally, most of the cells are concentrated in the area of nanopillars and grow in an orderly manner.

**IV. CONCLUSION**

In summary, the hybrid micro/nano structures fabricated by combining laser marking technology with metal assisted chemical etching (MACE) were introduced to study their effects on SHSY5Y cells. The results suggest that cells on the SiMP arrays (unetched substrate) have longer cell lengths and orderly arrangements, as well as tight connections with each other. While on the hybrid SiMP/SiNP arrays (etched substrate), the cells are trapped and they grow independently.
Specifically, about 90% of the cells show a preference for growing on the SiNPs, and they are elongated along the direction of SiMP arrays with relatively short cell lengths, as well as displayed a preference for independent growth. The findings provide the possibility of using hybrid micro/nano structures to regulate the cell behaviors, which is useful in tissue engineering.

ACKNOWLEDGMENT

This work was supported by National Key R&D Program of China (No. 2017YFE0112100), National Natural Science Foundation Program of China (Nos. 62175020, 61964007 and 62175019), EU H2020 Program (MNR4SCell No.734174; NanoStencil No. 776285), Jilin Provincial Science and Technology Program (Nos. 2020C022-1, 20190201287JC, 20190702002GH and 20200901011SF), Jilin Province Education Department Program (No. JJKH20210833KJ), and “111” Project of China (No. D17017).

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