Neuronal Growth on a-Si and Au Nanopillars

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ABSTRACT

Neuronal patterning is useful for understanding signal propagation between neurons as well as for biosensors and cell-based assays. The patterning of living cells has been made possible by employing surface physicochemical and topographic features. This study investigated neuronal growth on patterned nanopillars. Rat cortical neurons were cultivated on quartz substrates with amorphous silicon (a-Si) and Au pillars 100 and 500 nm in diameter. The neurites grew better with the larger diameter pillars, and the partly-selective neurite growth was observed for a-Si pillars but not for Au pillars. These results reveal the possibility of controlling neuronal growth by using a-Si nanopillars.

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1. Introduction

The guidance of neuronal axons and/or dendrites in an organized pattern in vitro is useful for examining the neuronal growth mechanism and neuron-matrix interactions, which are also important for understanding the signal propagation mechanism between neurons, and thus elucidating neuronal functions. Neurons arranged in specific patterns can also form an organized structure, such as neural network for the development of biosensor technology and tissue engineering applications.

In the past few decades, many studies have been undertaken with the aim achieving neuronal guidance using surface topographic features and/or physicochemical properties.1,2 Among several different topographies, micrometer sized pillars have been used for different purposes such as to promote neuronal axonal growth and orientation3–5 and cellular differentiation.5–8 Smaller diameter pillars, nanopillars, are applied for different purposes, for example, examination of neuronal development9 and pinning neurons.10–12 Nanopillars have also been used to access cellular membranes for electroporation,13,14 which led them to the successful measurement of neuronal intracellular action potentials.15,16 Although these approaches intended to achieve new biological interfaces, there have not been much studies related to nanopillars used as scaffolds. Moreover, detailed morphologies of neurites grown on the nanopillars have not been examined. In this study, the nanopillars are used as a scaffold on the top of which neurons grow, and a scanning electron microscopy (SEM) was employed to examine the neurite morphologies. Here, we investigated neuronal growth on amorphous silicon (a-Si) and gold (Au) nanopillars with diameters of 100 and 500 nm.

This detailed knowledge about the optimal condition for neuronal scaffolds such as pillar size and material will be useful for achieving neuronal guidance. This knowledge will also be used to establish biosensors and a bioassay platform. This semi-three-dimensional cultivating platform can also provide the neurons with a unique environment in which to connect to each other in a three-dimensional manner, and a novel biological interface will be thus achieved.

2. Experimental

2.1 Nanopillar substrate

Nanopillars were fabricated on quartz substrates using electron beam lithography as shown in Fig. 1. The pillars were 500 nm high, and either 100 or 500 nm in diameter, and the distance between them was either twice or four times the pillar diameter. The pillar material was either amorphous silicon (a-Si) or gold with titanium as a binder. The a-Si pillar substrates were cleaned with piranha solution (H2O2:H2SO4 = 1:3) and briefly etched with NH4F just before the next process, which was used to remove the oxidized silicon layer. The Au pillar substrates were cleaned using oxygen plasma.

2.2 Neuronal culture

Cortical neurons were obtained from Wistar rat (embryonic 18 days), and cultivated on a nanopillar substrate for 2 to 7 days in a neurobasal medium with L-glutamine, glutamate, gentamycin, and B27 supplement, at 37°C in 5% CO2.

Figure 1. Fabrication of nanopillar substrate using electron beam lithography.
2.3 Sample observation

After cultivation, the samples were fixed with paraformaldehyde (PFA) and glutaraldehyde, and with osmium tetroxide. The samples were then dehydrated with ethanol and finally with 100% tert-butanol. The samples were stored at −20°C, and then dried at −50°C for 3 h to prevent crystallization (Tokyo Rikakikai Co. Ltd.). The samples were observed with SEM (S-4300SE, Hitachi High-Technologies Corp.). For immunostaining, the samples were fixed with PFA and blocked. The cells were then incubated with Cy3-conjugated Pan Neuronal Marker antibody (1:100 dilution, Merck Millipore) before being observed with a confocal laser scanning microscope (LSM510, Carl Zeiss).

3. Results and Discussion

3.1 Pillar dimension

First, we examined pillar sizes and distances. Figure 2 shows SEM images of neurites cultivated on a-Si nanopillar substrates for 2 days in vitro (DIV). Neurites were successfully observed as developed on the structure with different diameters and distances. We also observed some neurites on the base substrate (quartz) away from the pillars as shown by the arrow in Fig. 2(b). This is probably because the neurite did not adhere to the next pillar and did not grow on the pillars.

We also noticed that the 100 nm diameter pillars were bent as shown by the arrows in Fig. 2(c, d). This was due either to neuronal attachment or elongation or to cellular movement during development. The effect of treating the cultivated neurons for SEM observation cannot be denied, however, these bends indicated that lateral force had been exerted to the pillars. This suggests that neurites adhered to pillars, indicating that nanopillars can be scaffolds for neuronal growth and patterning.

Then to examine neurite characteristics, we measured their widths on the nanopillars as shown in Fig. 3. This shows that the neurites grown on 500 nm diameter pillar were as wide as the neurites on a quartz substrate tested as references. It indicates that a diameter of 500 nm is large enough for neurite growth. In contrast, the neurites were thinner on the 100 nm diameter pillars. These neurite widths were not affected by the pillar distance.

This phenomenon could be explained by the size of adhesion area. Neurons adhere to an extracellular matrix (ECM) using certain areas where large integrin-rich protein complexes are expressed at the basal surface, which is called focal adhesion (FA). FA physically connects ECM to the cytoskeletons in neurons. However, the neurites adhered to 100 nm diameter pillars in this study probably do not have large FA nor grow wider.

The thin neurites on the 100 nm pillars would not be in ideal condition for neurons because the tips of the neurites, or growth cone, increase in width during development. Moreover, when dendrites became thinner, the probability of synaptic connections to other neurons and synaptic efficiency both decrease because of increased impedance in the neurites. Likewise, the substrate surface condition such as its structure is important in terms of the morphological properties of neurons in vitro and also as regards functional behavior. Given that the neurites on 500 nm diameter pillars had a similar width to that on quartz, a diameter of 500 nm is an appropriate size for elongating the neurites and for implementing neuronal functions.

3.2 Pillar material

We then investigated the difference between the neuronal preferences in the nanopillar materials, a-Si and Au. Figure 4 (A1 and B1) shows fluorescent images of neurons on a-Si (A1) and Au nanopillars (B1) at 6DIV. The neurons, stained red, on a-Si nanopillars were observed to grow randomly but partly following the pillar pattern as shown by the arrow in A1. In contrast, the neurons on the Au nanopillars grew completely randomly. These images show that neurites had a greater preference and grew selectively on a-Si, than on Au nanopillars. Figure 4 also shows an example of SEM images of neurites relative to the nanopillars of a-Si (A2, 3) and Au (B2, 3) at 2DIV. The neurites on Au nanopillars had a greater tendency to become attached to the quartz substrate, as shown by the arrow in B3. This corresponds to the result obtained in Fig. 4 (A1, B1).

For an accurate examination, we performed a statistical analysis. Figure 4(C) shows the ratio of the neurite tip position on the nanopillars relative to the quartz substrate at 2DIV. For a-Si, the preference exceeded 80% for nanopillars but was below 60% for Au nanopillars. These differences suggest that neurites were selectively attached and grew better on the a-Si pillars.

SiO₂, Si and gold are all biocompatible, however, a focused ion beam/scanning electron micrograph (FIB/SEM) observation showed that neurons have no affinity with gold. By contrast, SiO₂ and Si are commonly used as materials for neuronal platforms in vitro, although there have been few reports about pillar materials. These findings correspond to our pillar material preference result.
4. Conclusion

This study demonstrated the possibility of controlling neuronal growing conditions using nanometer-scale pillars made of a-Si as scaffolds. The pillar size will be optimized with a diameter of 500 nm.

These techniques will provide a semi-three-dimensional environment for neurons in which to examine the cellular-molecular interactions between neurons and the environment. Such work will help to implement a new platform for biosensors and bioassays. This approach will also realize a new biological interface for examining the neuronal developing mechanism in vitro.

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References