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Atomic force and confocal microscopy for the study of cortical cells cultured on silicon wafers

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Abstract The primary cortical cells were selected as a model to study the adherence and neural network development on chemically roughened silicon substrates without any coatings using confocal laser scanning microscopy (CLSM) and atomic force microscopy (AFM). The silicon substrates have a nano-range roughness (RMS) achieved by chemical etching using hydrofluoric (HF) acid. After 7 days of culturing, the neurons were observed to connect together and form dense neural networks. Furthermore, AFM results revealed that some porous structures at a few micrometer range existed between the neuron cells and the silicon substrates. It is suggested that the porous structures are made of extracellular matrix (ECM) components and play an important role in the neuronal adhesion and neurite outgrowth on the inert silicon wafers.

Introduction

The development of intelligent implants and bioneural networks requires the integration of silicon based electronics and biological systems. Silicon has been widely used in such in vitro and in vivo applications as substrates in the last decade [1–5]. Previous studies have shown that silicon material does not exhibit a significant amount of cytotoxicity and it is suitable for the manufacture of implantable devices and cell-based biosensors [6–9]. Because the original surface of silicon and silicon oxide is not suitable for the neural cell adhesion, some proteins or macromolecules, e.g. polylysine, were used as coatings in order to improve the cell adhesion in most of the previous studies [10]. Recent studies found that if there is no coating, the neural cells can still adhere and spread on the silicon substrates with the proper microstructure and surface morphology, e.g. the nano-structured porous silicon and the roughened silicon wafers with nano-scale roughness [11–16].

In the present work, we have cultured the primary cortical cells on the chemically roughened silicon wafers and characterized the cellular morphology and spatial relationships among the neurons, glial cells and silicon substrates. Furthermore, the surface topograph of the silicon substrates after the culturing was compared with that before the culturing in order to find the reason for the improvement of neural cell adhesion.

Materials and methods

Silicon wafers

The polished surface of silicon (111) wafers (the Institute of Beijing Nonferrous Metal, China) was used as the original front side. The wafers were cut into square pieces (1 × 1 cm) and then processed according to Fan's protocol [14, 15]. After ultrasonic washing them in acetone, the wafers were etched in 20% HF

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solution for 40 min. Before cell seeding, the wafers were immersed in 70% ethanol for 2 h or sterilized using O_3 gas for 40 min without any further treatment.

Cell culture

Primary cultures of cortical neurons were prepared from E15 Sprague–Dawley rats as previously described [17–19]. All cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM, Gibco, CA) supplemented with 10% fetal bovine serum and penicillin/streptomycin at 37°C in a humidified incubator with 5% CO_2. Silicon substrates were seeded with about 1×10^5 cells each in 1 mL of culture medium. Afterwards, the culture medium was changed every 24 h. Cytosine arabinoside was added to prevent the growth of nonneuronal cells in a final concentration of 10 mM after 48 h of culturing.

Microscopy analysis

The CLSM system (Radiance 2100, Bio-Rad, UK) was used in the investigation of the neural network on silicon wafers. In the fluorescence mode, propidium iodide (PI) staining was performed to visualize the cells on the opaque silicon substrates while the reflection mode was introduced to reinforce the results. The protocol of PI staining was modified from the methods described in the previous literature [20]. Cells were incubated in 5 µg/mL PI (Sigma, USA) solution diluted in PBS buffer for 20–30 min at 37°C. Then the samples were washed in PBS buffer 3 times and mounted on glass slides for observation. The mixture of 50% glycerin and 50% PBS buffer was added to keep the samples wet during the examining period. According to the emission wavelength of PI, the filter was set 570LP and the laser was 543 nm in the fluorescence mode. In the reflection mode, the laser was 488 nm and the filter was set 488/10 nm.

The AFM investigations were performed with a commercial AFM instrument (Picoscan, MI, USA), using a rectangle silicon cantilever (CSC12, Silicon-MDT, Russia, 300 µm length, spring constant, as indicated by the supplier, 0.01–0.1 N/m). A 120 × 120-µm scanner was used for the cells. Because of the z-range limitation of AFM scanner, the observed cells were fixed and dehydrated gradually. The samples were prepared as described in previous literature [21]. The neural cells cultured for 5–7 days on silicon wafers were fixed for 30 min in 4% formaldehyde in normal saline. After wash in triply distilled water, the cells were dehydrated in ethanol series 75, 90 and 100%. Then the silicon wafer was immobilized by double face glue tape onto the steel sample stage. AFM imaging was carried out in contact mode at room temperature in the air. From the AFM results, we can distinguish the neurons and glial cells by the height of the cells [22].

Results

Topograph of the silicon wafers

The original polished surface of untreated silicon wafers is very smooth. Using AFM, we obtained the topographic image of the silicon wafers after etching in HF solution, e.g., as shown in Fig. 1. The scan range was 5 × 5 µm square. From the height data, the statistical roughness of the silicon wafers was 54.9 ± 15.0 nm (RMS, n = 6). Some protuberant crystals were observed on the surface with the diameter size ranging from 50 nm to 500 nm in Fig. 1. This kind of surface topograph may be a suitable microenvironment for the protein deposition, the neural cell adhesion, neurite outgrowth and neural network formation.

Neural networks

Dissociated rat neural cells were seeded onto the silicon substrates with nano-scale surface roughness. As expected, the cortical neurons were found to survive on the roughened silicon wafers. The attachment of these primary neural cells seemed to depend on the serum used in the culture medium and seeding method. The attached neurons developed long neurites during the first 3 days of culturing, and formed dense, interconnected networks within 7 days post-plating.

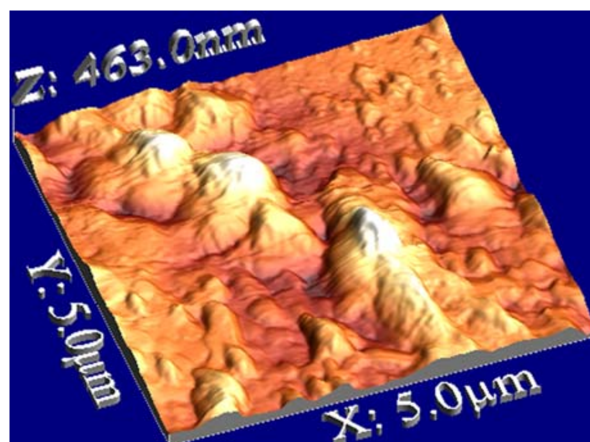

Fig. 1 AFM three-dimensional topographic image of the surface of the silicon wafer after etching

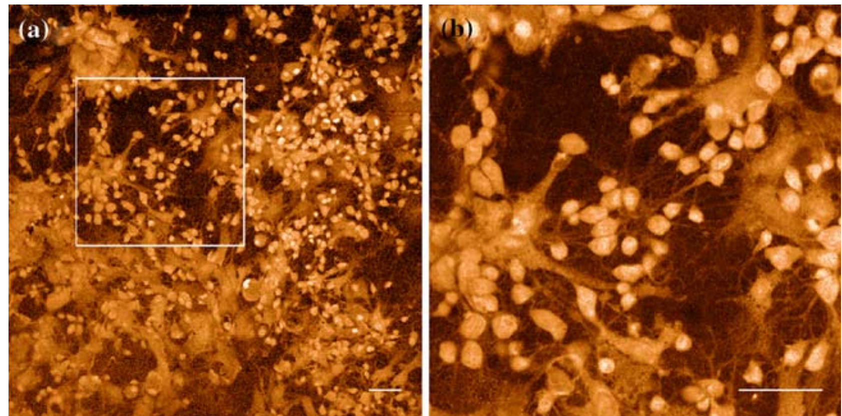

Fig. 2 Fluorescent images of neural cells cultured on the silicon wafers after 3 days. (**b**) is the magnification of part of (**a**) indicated by a square. The bars are 50 μm

Figure 2 shows the confocal fluorescent images of the cortical cells after 3 days of culturing. It is observed that the flattened glial cells were overspreading on the whole silicon substrates and even covered most areas of the wafers. The cellular bodies of neurons were distributed near the glial cells and some neurons were grown onto the cellular membrane of glial cells. Meanwhile, the neurons were found to prefer growing together and forming cell clusters around the glial cells as shown in Fig. 2b. From the confocal reflected images, we could clearly observe more details about the morphology of the neurons than the fluorescent images because PI only stained the main cellular components. Figure 3 shows the cells after 3 days (a) and 7 days (b) of culturing. In the first 3 days, the neurites of more than 200-μm long were extended from the neurons as shown in Fig. 3a. After 7 days, many more neurons and neurites developed in cultures as shown in Fig. 3b. The neurons were tightly connected each other with a large number of dendrites and synapse-like filaments. Because of additional arabinoside in the culture media, the number of glial cells reduced to a very low level compared to that in the first 3 days. However, some glial cells with flattened cellular body can be observed beneath the neuronal networks in Fig. 3b.

Spatial relations among the neurons, glial cells and substrates

The neurons and glial cells were investigated in more details using AFM. Because the height and size of somata is very different between the two kinds of cells, they can be distinguished easily [22]. The measurements of cortical neurons show that they have a width of 8.9 ± 1.4 μm at the medium of the neuron somata and a height of 1.7 ± 0.4 μm (n = 10). The glial cells have a width of 20.7 ± 13.7 μm and a height of 0.27 ± 0.16 μm (n = 6).

From the AFM results, it is found that some cortical neurons adhered onto the membrane of glial cells as shown in Fig. 4, which is accordant with the CLSM results. The neuron shows typical bipolar neurites of more than 30-μm long. The soma height of the cortical neuron is measured more than 1.4 μm as shown in profile (a) of Fig. 4. And the height of the nearby glial cell is only about 0.2 μm. The cortical neuron has a width of about 10 μm at the medium of the cellular

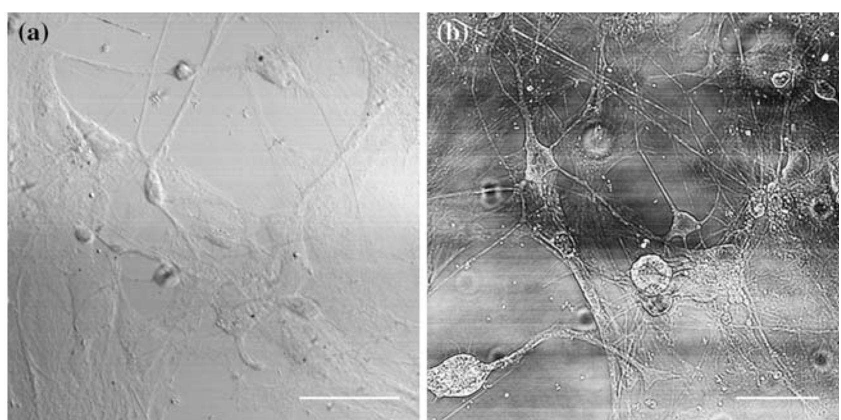

Fig. 3 Reflection images of neural cells cultured on the silicon wafer after (**a**) 3 days (**b**) 7 days. The bars are 50 μm

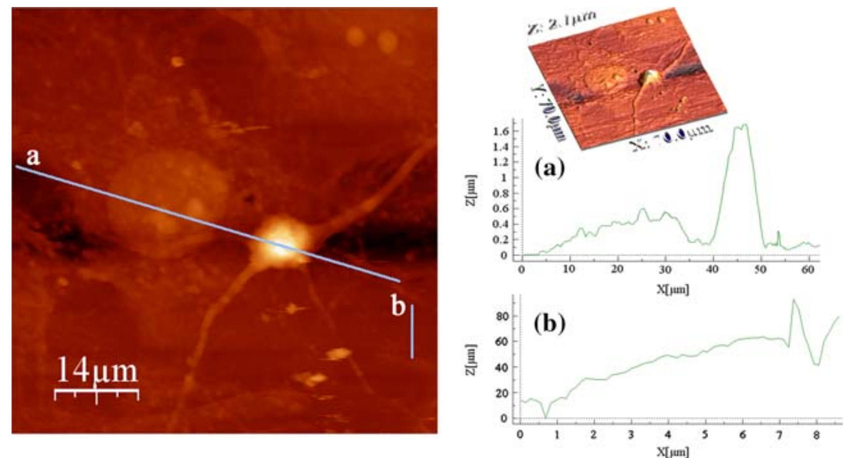

Fig. 4 AFM images of neural cells cultured on the silicon wafer after 7 days of culturing. The upper right insert is the 3-dimensional reconstruction image. The profiles were corresponding to the two line segments in the left image: (**a**) the cross-section through the somatas of the two cells, (**b**) the cross-section of a culture-less area

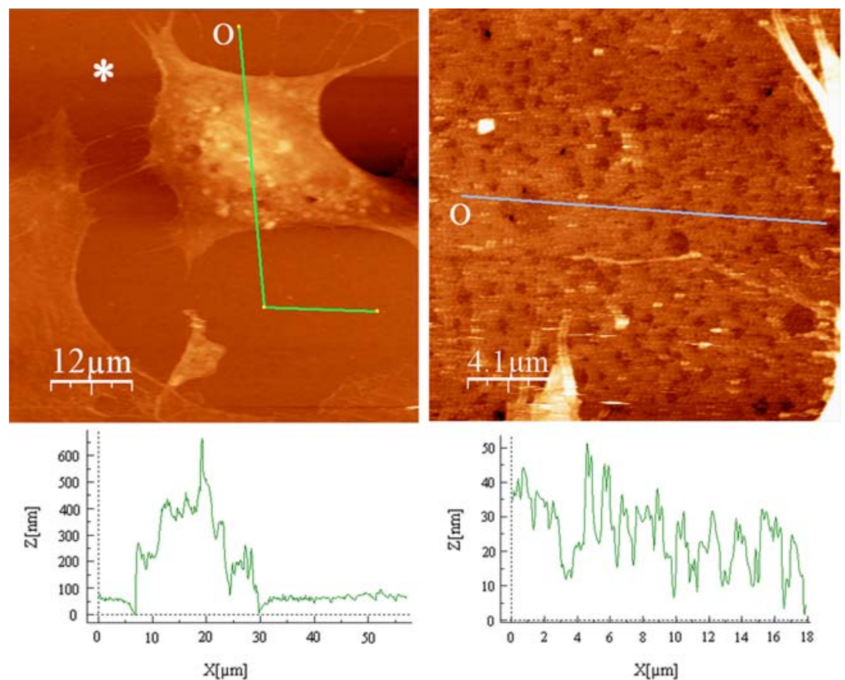

Fig. 5 AFM images of a single neuron. Porous structures (**b**) are near the cellular body indicated by asterisk in (**a**). The profiles were corresponding to the line across the above images. Both of the lines begin from left indicated by capital letter “O”

somata, while the glial cell beneath the cortical cell has a width of about 20 µm. Meanwhile, the height of culture-less areas was ranging from 15–60 nm as shown in profile (b) of Fig. 4. The surface of silicon wafers seemed unchanged after cell culturing.

We focused on some single neurons directly on silicon wafers as shown in Fig. 5a. The diameter of the somata is about 30 µm and the height is more than 600 nm. Its morphology is different from that of the neurons near the glial cells. We have carefully investigated the area near the cellular pseudopods indicated by asterisk. The surface shows some porous structures as shown in Fig. 5b. The diameter of the pores is about 1–3 µm. It is inferred that such porous structures should also exist between the cellular membrane and the silicon substrate.

Discussion

From the above results, the primary neurons can survive with the glial cells and form dense neural networks on the chemically roughened silicon wafers without any coating. Some glial cells were found to grow beneath the neural networks as a carpet. The present results confirmed the previous studies that the topographical modification of silicon wafers can improve neuronal adhesion [14, 15]. In addition, the method of chemical etching is very simple and easy to achieve. It will not bring extra influence to the interface between the cell and the silicon surface. If the silicon wafers were coated with proteins, the gap between the cells and silicon substrates will be 90 nm and 40 nm for laminin and polylysine, respectively [9].

If there is no coating, the effect of gap will not exit. Furthermore, it has the same advantage for cell adhesion but simpler procedures than that of the porous silicon [11, 13].

It is noticed that the dense neural networks formed with a small quantity of glial cells after 7 days of culturing. It is very interesting some neurons were found to grow onto the membrane of glial cells. It is solid evidence that the glial cells must be related to the neurons on the silicon wafers in this condition. However, the relationships between the neurons and glial cells are not very clear. In the previous studies, glial cells were found to be either neurotrophic or neurotoxic depending upon the microenvironment in vitro [23]. On the roughened silicon wafers, the glial cells seemed positive for the neurons from the present results. However, it needs more evidences about the connecting synapse, neural transmitter and so on. The normal electrical activity indicates the function of neurons. The electrical properties of the neurons will be affected when the substrates and culture medium are different [24]. The electrical investigations such as patch clamp will be needed to reveal the functional relationships in further studies.

The porous structures on the silicon wafers are suggested to a key factor for the attachment of cortical neurons on such inert surfaces. Similar results have been reported for the hippocampal neurons cultured on the roughened silicon wafers [25]. In the previous study, it is observed the porous structures on the silicon wafers after ultrasonic wash in ethanol. In this manuscript, the porous structures were observed in situ near the cellular body on the silicon substrates without washing. From the comparison between the silicon surfaces before and after culturing, it is inferred that the neural cells secreted the ECM components forming the porous structures during the culture periods, because there were not such similar porous structures before cell seeding. The neural cells could survive on the roughened silicon wafers through modifying the surface microenvironments within a certain range. The roughened silicon wafers are suggested to be able to promote the deposition of ECM proteins and form the porous structures at a few micrometer ranges.

It is still difficult to clearly describe the complex interface between the neurons and silicon substrates from the present results. However, the AFM results offered some useful information that the cells could change and control the interface themselves in a certain extent.

Conclusion

The primary cortical neurons were cultured on the chemically roughened silicon wafers without any coating and investigated to form highly interconnected neural networks. It is revealed that some porous structures existed on the silicon substrates and they can support the cell adhesion during culturing. The results will be positive to the manufacture of silicon chips for intelligent implants and neuron-based biosensors.

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References

1. P. MANOS, J. J. PANCRAZIO, M. G. COULOMBE, W. MA and D. A. STENGER, *Neurosci. Lett.* **271** (1999) 179
2. S. TATIC-LUCIC, J. A. WRIGHT, Y. C. TAI and J. PINE, *Sensors Actuat. B-Chem.* **43** (1997) 105
3. D. A. STENGER, G. W. GROSS, E. W. KEEFER, K. M. SHAFFER, J. D. ANDREADIS, W. MA and J. J. PANCRAZIO, *Trends Biotechnol.* **19** (2001) 304
4. S. L. SMITH, J. W. JUDY and T. S. OTIS, *J. Neurosci. Methods* **133** (2004) 109
5. G. BUZSAKI, *Nat. Neurosci.* **7** (2004) 446
6. S. C. BAYLISS, P. J. HARRIS, L. D. BUCKBERRY and C. ROUSSEAU, *J. Mater. Sci. Lett.* **16** (1997) 737
7. S. VASSANELLI and P. FROMHERZ, *Appl. Phys. A-Mater. Sci. Process.* **65** (1997) 85
8. S. VASSANELLI and P. FROMHERZ, *Appl. Phys. A-Mater. Sci. Process.* **66** (1998) 459
9. G. ZECK and P. FROMHERZ, *Langmuir* **19** (2003) 1580
10. D. H. DAVIS, C. S. GIANNOULIS, R. W. JOHNSON and T. A. DESAI, *Biomaterials* **23** (2002) 4019
11. S. C. BAYLISS, L. D. BUCKBERRY, I. FLETCHER and M. J. TOBIN, *Sensors Actuat. A-Phys.* **74** (1999) 139
12. S. C. BAYLISS, L. D. BUCKBERRY, P. J. HARRIS and M. TOBIN, *J. Porous Mater.* **7** (2000) 191
13. S. B. DE LEON, A. SA'AR, R. OREN, M. E. SPIRA and S. YITZCHAIK, *Appl. Phys. Lett.* **84** (2004) 4361
14. Y. W. FAN, F. Z. CUI, S. P. HOU, Q. Y. XU, L. N. CHEN, I. S. LEE, *J. Neurosci. Methods* **120** (2002) 17
15. Y. W. FAN, F. Z. CUI, L. N. CHEN, Y. ZHAI, Q. Y. XU and I. S. LEE, *Appl. Surface Sci.* **187** (2002) 313
16. R. A. KRUSE, S. S. RUBAKHIN, E. V. ROMANOVA, P. W. BOHN and J. V. SWEEDLER, *J. Mass Spectrom.* **36** (2001) 1317
17. M. S. EVANS, M. A. COLLINGS and G. J. BREWER, *J. Neurosci. Methods* **79** (1998) 37
18. J. E. HUETTNER and R. W. BAUGHMAN, *J. Neurosci.* **6** (1986) 3044
19. C. E. JAHR and C. F. STEVENS, *Nature* **325** (1987) 522
20. C. BRANA, C. BENHAM and L. SUNDSTROM, *Brain Res. Protoc.* **10** (2002) 109

21. Y. G. ZHANG, X. JIANG and L. SUN, *J. Struct. Biol*. **144** (2003) 327
22. Y. YAMANE, H. SHIGA, H. ASOU, H. HAGA, K. KAWABATA, K. ABE and E. ITO, *Arch. Histol. Cytol.* **62** *(1999) 355*
23. S. C. ZHANG and S. FEDOROFF, *Acta Neuropathol. (Berl)* **91** (1996) 385
24. W. J. PARAK, M. GEORGE, M. KUDERA, H. E. GAUB and J. C. BEHRENDS, *Eur. Biophys. J*. **29** (2001) 607
25. J. MA, B. F. LIU, Q. Y. XU and F. Z. CUI, *Colloids Surf B Biointerfaces* **44** (2005) 152