Transactions of the Materials Research Society of Japan 32[4] 917-920 (2007)

Modification Limit in Line Width of Carbon Negative-Ion Implantation to Polystyrene Surface for Nerve-Cell Adhesion and Neurite Outgrowth

Hiroshi Tsuji, Piyanuch Sommani, Mitsutaka Hattori, Tetsuya Yamada, Hiroko Sato, Yasuhito Gotoh and Junzo Ishikawa
Department of Electronic Science and Engineering, Kyoto University, Nishikyo-ku, Kyoto 615-8510, Japan
Fax: 81-75-383-2283, e-mail: tsuji@kuee.kyoto-u.ac.jp

We have investigated about limitation of line width in surface modification of polystyrene for nerve cell adhesion and neurite outgrowth by carbon negative-ion implantation. Carbon negative ions were implanted into polystyrene film on glass at 10 keV with 3 x 10^{15} ions/cm^2 through a double-mask set with array of ridge shaped apertures gradually changing the width from 50 to 0 nm. The polystyrene film was prepared three times by spin coating with 7% PS in toluene at a speed of 1,000 rpm. After seeding on the modified SCPS sample, nerve-like cells of PC-12h were cultured with *DMEM medium* including 10% serum for 2 days in an incubator with 5%CO_2 air flow at 37°C. Then, the cells were cultured for more 2 days in the incubator after replacing the serum DMEM medium to serum-free DMEM. As a result, the cell body adhered on the ion implanted area with the minimum width of 7 nm, and neurite outgrowth was observed also observed only on the ion implanted area until the width of 5 nm..
Key words: Surface modification, Negative ion implantation, Cell adhesion, Neurite outgrowth, Polystyrene

1. INTRODUCTION

Surface modification of polymeric materials by ion implantation is very interesting treatment method for improving biocompatibility, since the treated surface becomes hydrophilic and has considerably good cell adhesion property by using ion implantation with not only conventional positive ions [1-3] but also our negative ions [4-13]. The negative ion implantation has a "charge up free" property for insulators such as many polymeric materials, so that we can execute the implantation with a precise energy and dose without any charge compensator [14-16]. application of this surface treatment by ion implantation, especially, carbon negative-ion one at a relatively low energy of about 10 keV, the optimal condition of the ion implantation should be cleared. We investigated about ion energy and dose amount in the surface treatment of polystyrene and silicone rubber for cell adhesion of nerve-like cells of PC-12h (rat adrenal pheochromocytoma) [11, 12] and MSC (rat mesenchymal stem cells) [13]. There still remains a problem in pattering of the surface by the ion implantation. It is optimal or minimum line width of the pattering. In this paper, we have implanted carbon negative ions through a double-mask which has periodical wedge-like apertures. And by using this patterning surface, we have investigated line widths of carbon negative-ion implantation required for cell adhesion and/or neurite extension on the treated surface.

2. SURFACE PATTERING BY NEGATIVE- ION IMPLANTATION

2.1. Pattering mask

We used two metal masks having an array of rectangular apertures of 50 μm width and 5 mm in length and 70 μm spacing to form wedge-like apertures. When two masks were superimposed with a very little angle of about 0.6 – 1.0 degree, an array of periodic wedge-like apertures with a wide width of 40 μm, the minimum one of almost 0 μm and 3 – 5 mm in length is obtained. The periodic length was 120 μm. The schematic configuration of this double mask is shown in Fig.1. The slope of one wedge shape is very slow of 1/60 – 1/100, so that the difference of the width between both sides of one PC-12h cell (20 μm at maximum) is about 0.4 μm. We can evaluate the required width by the positions of cell adhesion and extended neurite.

2.2 Carbon negative-ion implantation

We prepared a spin-coated polystyrene (SCPS) film on a glass plate (24 mm x 24 mm, 0.5 mm in thickness, Corning 7059) by using a solution of polystyrene in toluene at 7 wt.% at a spinning speed of 1000 rpm at three times. The thickness of SCPS film coated at three times was about 700 nm.

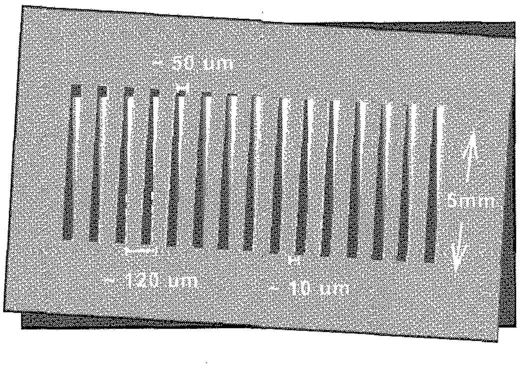

Fig.1. Schematic diagram of double mask to make an array of wedge-like apertures. The single mask has an array of rectangular apertures of 50 μm width and 5 mm in length. As superimposing two masks at a little angle of 0.6 - 1 degree, wedge- like-aperture array is obtained. The minimum width is almost zero.

Carbon negative ions were implanted into the SCPS film on glass through the double mask at various energies in a range of 5 - 20 keV with a constant dose of 3.0×10^{15} ions/cm^2 in a residual gas pressure less than 1×10^{-4} Pa by a negative ion implanter equipped with a sputter-type heavy negative-ion source [17, 18]. The implantation was executed with a small current density less than 400 nA/cm^2 in order to avoid surface melting of polystyrene. This implantation condition of dose was selected from our previous works [4-13].

3. CELL CULTURE AND OBSERVATION

Each of the carbon negative-ion implanted SCPS samples was pasted on a disposable polystyrene dish (non-treated PS dish, 35 mm in diameter, Corning, USA) by silicone glue. After drying the glue, the sample dishes were sterilized with 70 % ethanol followed by rinsing three times with de-ionized water and one time with phosphate buffered saline (PBS). Then, nerve-like cells of PC-12h (rat adrenal pheochromocytoma) with a response property to the nerve growth factor were seeded on the sample dishes and a control dish of collagen (type I)-coated PS dish at 1.2×10^5 cells/ml for each, and cultured in a medium of DMEM (Dalbecco's Eagle's Modified Medium, Nissui Co. Ltd, Japan) including 5% horse serum (HS, Biomedicals, USA) and 5% fetal bovine serum (FBS, Bio-Wittker, USA) with antibiotics for 2 days in an incubator at 37 oC with 5% CO_2 air flow. After replacing the culture medium to serum-free DMEM for cell growth and adhesion, we continued to culture the cells in the same incubating conditions for 2 days more for neurite extension from cell body.

After cultivation for 4 days in total, we observed cells cultured in the sample dishes with optical phase-contrast microscope (CK-2, Olympus, Japan). In the pattern, the periodic length of 120 μm is constant in whole area. The implantation line width can be calculated based on the periodic length.

4. RESULTS

4.1 Cell Adhesion Properteis

The PC-12h cells and neurite outgrowths cultured on the carbon-implanted SCPS surface with fine pattern at 10 keV with 3.0×10^{15} ions/cm^2 are shown in Fig. 2: (a) area of a relatively wide line width of about 20μm, (b) of a narrow width less than 10 μm in the pattern implantation, and (c) on collagen of the control. We can find adhesion of a lot of cells and neurite outgrowth on the wider area in Fig. 2(a), where the adhered cell density is almost same that on collagen type-I of the control. The cells also adhered on the width narrower than cell size such as 5 μm. As for other implanted sample at different energies, almost same tendency was obtained. We evaluate this optimal width for implantation by the average width of the modified lines, where a lot of cells adhered in a group. The minimum line width required for cell adhesion or neurite outgrowth was different, because their sizes were different almost by ten times.

The narrowest width required for a lot of cell adhesion on the modified surface is considered as

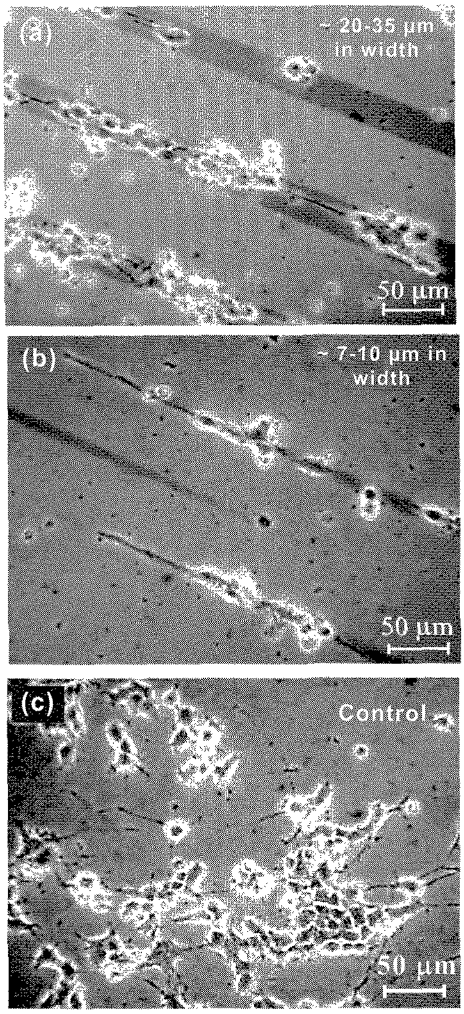

Fig. 2. Phase contrast micrographs of adhered PC-12h cells and extended neurites on various surfaces: (a) in wide and (b) narrow regions of implanted wedge-like pattern of polystyrene with 3×10^{15} ions/cm^2 at 10 keV and (c) on collagen (type I) of the control after culturing for 4 days.

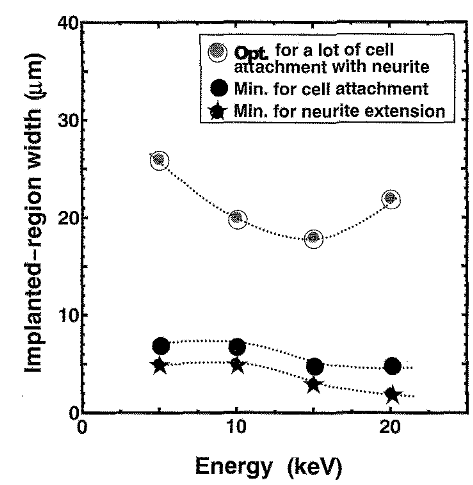

Fig. 3. Adhered line width of cells and neurites on the wedge-like pattern on SCPS implanted by Carbon negative-ion implantation

the optimal width for ion-implantation treatment. We measured the optimal width for group cell adhesion and the minimum widths for one cell adhesion and for neurite extension. Fig. 3 shows the optimum and the minimum line widths of the wedge-like pattern for the various samples modified at different energies of ion implantation. The optimum width was a range of 18 – 26 μm and is considered to demand more than cell size. The better modification energy of carbon negative ions is considered to be around 15 keV.

The minimum widths for cell and neurite adhesion are both decreased a little with an increase in ion energy. But it seems that they are both saturated in the modification energy more than 15 keV.

4.2 Neurite outgrowth Properties

The neurite length extended on the C-implanted pattern was measured on various modified surfaces as well as on the control after 4 days in cell culture. Fig. 4 shows the maximum and average lengths of extended neurites on the wedge-like pattern. In the figure, the maximum and average neurite lengths of cells cultured on the control surface of collagen are also shown by solid and dotted lines, respectively. The maximum and average lengths both showed a little increasing tendency as an increase in ion energy of implantation. However, the neurite lengths on the modified surface were shorter than those on the control of collagen.

5. DISCUSSION

5.1. Cell Adhesion on Narrower C-implanted Region

Cell adhesion on modified surface by C-implantation is considered to be due to improvement in hyidrophilicity of the surface. By the implantation modification of polystyrene, the surface adsorbed more the extracellular matrix (ECM) [6]. In cell adhesion on substrate, in general, adhesive proteins such as laminin, fibronectin, vitronectin and collagen of ECM are required on the surface as footing sites. In the research of ECM adsorption properties on C-implanted surfaces by XPS evaluation of adsorbed nitrogen atom of proteins, the ECM adsorption on polystyrene increased 8.5 % from 5.5 % by carbon negative ion implantation. This increase in ECM adsorption corresponded to the contact angle lowering, i.e., more hydrophilicity.

As shown in Fig. 2(b), the cells adhered even on the C-implanted region narrower than the cell size lapping unimplanted region. This means that cells can be immobilized on the substrate without sweeping away from the adhesion position when some of protein receptors of cell membrane are connected to proteins adhered on the surface as "footing" sites. Therefore, the minimum modification line-width of about 5 μm was obtained for cell adhesion of 18-μm-size PC12h on the C-implanted polystyrene surface.

5.2. Neurite Extension on Narrower C-implanted Region

The slightly increasing energy dependence in the maximum and average neurite extension properties was obtained for various C-implanted surfaces in the ion

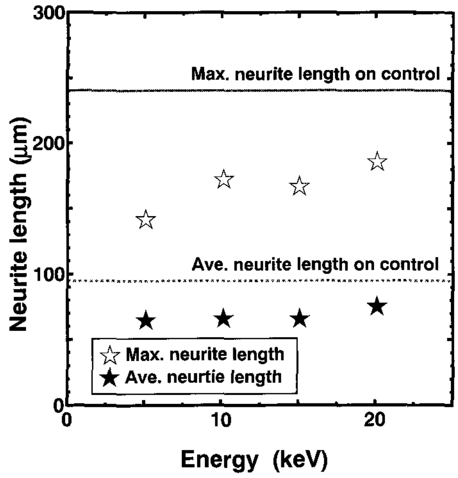

Fig. 4. The maximum and average neurite lengths from cell body adhered on the wedge-like pattern on SCPS implanted by Carbon Negative-Ion Implantation. Solid and dotted lines indicated the maximum and average neurite lengths of cells cultured on the control surface of collagen, respectively.

energy region of 5 – 20 keV in this experiment. This reason is also explained by the improvement of surface hydrophilicity and ECM adsorption as well as the cell adhesion in the narrower region mentioned in the previous subsection. As for neurite extension of NGF active PC12h cells, the NGF adsorbed on the C-implanted region more than the unimplanted ones.

In comparison with neurite extension on the control of collagen, the both of the maximum and average lengths on the modified polystyrene surface were shorter. In our latest research of neurite extension on C-implanted polystyrene with a rectangular slit array with constant width of 50 μm, the maximum length of extended neurite showed over 240 μm [11]. That length was almost same length on the control of collagen. Therefore, the reason of the shorter neurite extension on the narrower modified surface is considered to be due to limitation to the vector of neurite extension by the very narrow modification with wedge-like shape with width less than 50 μm narrower than the maximum length of 240 μm on the control. This very narrow width of the C-implanted surface prevented the cell to extend its neurite to all directions to make it shorter than that of its own ability.

6. CONCLUSION

The affect of modification widths of a range 0 – 50 μm on the polystyrene films by carbon negative-ion implantation on the cell adhesion and neurite extension of nerve-like PC12h were investigated by using a pattern mask consisting of an array of very steep wedge-shaped apertures. The both of cell adhesion and neurite extension were observed only on the C-implanted region. At the minimum for 15-keV implantation, cells adhered on the modified region of a width of 5 μm with lapping over the unimplanted surface much narrower than cell size of 18 μm. For the group adhesion of cells, the suitable modified width was about 20 μm, comparable with the cell size. The neurite extension was observed on the minimum modified width of about 2 μm. The suitable widths for guidance and immobilizations of cell body and the neurite extension were about 20 and 5 μm, respectively. The best energy condition for neurite extension on modified-line width was at 15 keV.

Acknowledgement
This work has been partially supported by the Grant-in-Aid for Scientific Research of MECSST, Japan, No.17360021.

References

[1] E. Leitao, M. A. Barbosa, K. De Groot, J. Materials Science: Materials in Medicine 9 [9] (1998) 543.
[2] H. Kawakami , K. Hiraka, S. Nagaoka, Y. Suzuki, M. Iwaki, J. Artificial Organs 7 [2] (2004) 83.
[3] Y. Yokoyama, T. Tsukamoto, T. Kobayashi, M. Iwaki, Surf. Coat. Tech. 196 [1-3] (2005) 298.
[4] H. Tsuji, H. Sato, T. Baba, S. Ikemura, Y. Gotoh, J. Ishikawa, Nucl. Instr. and Meth. B 166/167 (2000) 815.
[5] H. Tsuji, H. Sasaki, Y. Utsumi, H. Sato, Y. Gotoh, J. Ishikawa, Surf. Coat. Tech. 158–159 (2002) 620.
[6] H. Tsuji, H. Sasaki, H. Sato, Y. Gotoh, J. Ishikawa, Nucl. Instr. and Meth. B 191 (2002) 815.
[7] H. Tsuji, M. Izukawa, R. Ikeguchi, R. Kakinoki, H. Sato, Y. Gotoh, J. Ishikawa, Nucl. Instr. Meth. B 206 (2003) 507.
[8] H. Tsuji, M. Izukawa, Y. Utakawa, R. Ikeguchi, R. Kakinoki, H. Sato, Y. Gotoh, J. Ishikawa, Trans. Mater. Res. Soc. Japan 29 [2] (2004) 575.
[9] H. Sato, H. Tsuji, H. Sasaki, S. Ikemura, Y. Gotoh, J. Ishikawa, S. Nishimoto, CJPS. 22 [1] (2004) 69.
[10] H. Tsuji, P. Sommani, T. Muto, Y. Utagawa, S. Sakai, H. Sato, Y. Gotoh, J. Ishikawa, Nucl. Instr. and Meth. B 237 (2005) 459.
[11] P. Sommani, H. Tsuji, H. Sato, T. Kitamura, M. Hattori, Y. Gotoh, J. Ishikawa, Trans. Mater. Res. Soc. Japan 31 [3] (2006) 669.
[12] P. Sommani, H. Tsuji, H. Sato, T. Kitamura, M. Hattori, Y. Gotoh, and J. Ishikawa, Transactions of the Materials Research Society of Japan, 31, [3] (2006) 673.
[13] P. Sommani, H. Tsuji, H. Sato, M. Hattori, T. Yamada, Y. Gotoh, and J. Ishikawa, *The 16th Academic International Symposium of Materials Research Society of Japan*, K-04D, Dec. 8-10, Tokyo, Japan.
[14] H. Tsuji, Y. Toyota, J. Ishikawa, S. Sakai, Y. Okayama, and S. Nagumo, *Ion Implantation Technology-94*, (Eds: S. Coffa, G. Ferla, F. Priolo and E. Rimini, Elsevier, Amsterdam, 1995) p. 612.
[15] H. Tsuji, J. Ishikawa, S. Ikeda, Y. Gotoh, Nucl. Instr. and Meth. B 127/128 (1997) 278.
[16] H. Tsuji, Y. Gotoh, J. Ishikawa, Nucl. Instr. and Meth. B 14 (1998) 645.
[17] J. Ishikawa, H. Tsuji, Nucl. Instr. and Meth. B 74 (1993) 118.
[18] H. Tsuji, T. Taya, J. Ishikawa, and T. Takagi, *Proceedings of the International Ion Engineering Congress, ISIAT'83 & IPAT'83,* Vol. 1, (Ed, T. Takagi, Institute of Electrical Engineers of Japan, Kyoto, 12-19 Sept. 1983), p. 141

(Received December 10, 2006;Accepted May 15, 2007)