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Neurite Outgrowth Properties on Spin-coated Polystyrene Modified by Carbon Negative-ion Implantation through Pattern Mask

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The surfaces of the spin-coated polystyrene (PS) on glass were modified by carbon negative-ion implantation with various ion doses from $1x10^{14}$ - $3x10^{16}$ ions/cm^2 at various ion energies from 5 - 20 keV through a pattering mask slits of 50-μm width to investigate the neurite outgrowth properties. After 2-day *in vitro* culture of the nerve-like cells of rat adrenal pheochromocytoma (PC12h) on the C-implanted PS films, as well as on type-I collagen-coated dish as a reference, with the serum medium, the culture medium were changed to the serum free medium with a 50-ng/ml nerve growth factor (NGF) and the cells were cultured for observation of the differentiation in cell body and the neurite outgrowth. After 2-day culture in the serum free medium with NGF, results show that the neurite lengths on the surface of the PS implanted with around 2-3 ($x10^{15}$) ions/cm^2, corresponding to the optimum decreasing of contact angle, can be comparable to that of the surfaces of coated by type-I collagen.
Key words: negative ion implantation, neurite outgrowth, polystyrene, contact angle

1. INTRODUCTION

The negative ion implantation technique has been used to modify the surface of polymeric material for improved-attachment of nerve cells on the surface [1-6] because of the advantage of charge-up free [7-9]. As previous our work, the improved-attachment of cells on the polymeric surfaces modified by the carbon negative ion implantation is related to the modified physical surface property such as the hydrophilicity [3-4], and the contact angle of pure de-ionized water (DIW) is used to evaluate this surface property. The low contact angle corresponds to the high hydrophilic surface and to high possible attachment of cells. The dependences on the dose and energy implantation, on the circumstance and on the measuring time of contact angle in DIW were reported [3]. The researches related to the differentiation in nerve-like cells of rat adrenal pheochromocytoma (PC12h) such as nerve regeneration on the C-implanted polymeric surface [5] and neurite outgrowth on the Ag-implantation polymeric surface have been carried out [10]. However, the direct neurite-outgrowth work has not been investigated.

In this paper, the neurite outgrowth properties of nerve cell attached on the spin-coated polystyrene films modified by the carbon negative-ion implantation are studied.

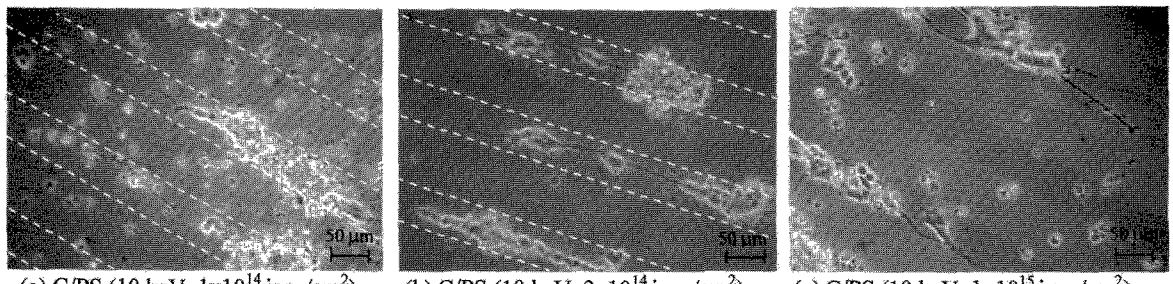
2. EXPERIMENT

Spin-coated polystyrene films on glass (7% of polystyrene in toluene, PS Nacalai Tesque Inc., Japan) were implanted by carbon negative ions for surface modification. Carbon negative ions produced in a cesium sputter-type heavy negative-ion source, a neutral and ionized alkali metal bombardment-type negative ion source (NIABNIS) [11, 12], were mass-separated and transported to an implantation chamber. The carbon negative-ion beam of 11.28 mm in diameter was implanted to the films at various ion energies from 5 to 20 keV and various doses from 0.1, 0.3, 0.7, 1, 2, 3, 5, 7, 10 and 30 (x 10^{15}) ions/cm^2 with a current density less than 400 nA/cm^2 under residual gas pressures less than 6 x 10^{-4} Pa. The polystyrene films were implanted through a pattering mask of many slit apertures 50-μm width and 70-μm spacing, and each C-implanted sample was then fixed with a 35-mm dish (Non-treated polystyrene dish, Corning). After 2-day dry all fixed dishes were sterilized by 70% ethanol, rinsed three times with the sterilized DIW and rinsed once with the phosphate buffered saline (PBS) before cell culture. Nerve-like cells of PC12h with $3.7x10^5$ cells/ml were cultured on the sample dishes in Dulbecco's modified Eagle's medium (DMEM, Nissui, Japan) containing 5% heat-inactivated horse serum (HS, Biomedicals, USA) and 5% fetal bovine serum (FBS, Bio-Wittker, USA), sodium hydrogen carbonate (1.8 mg/ml, Wako, Japan) with antibiotic of penicillin G and streptomycin for 2 days under 5% CO_2 at 37°C in incubator, as well as on type-I collagen-coated dish as a reference. Then, the culture medium was substituted by the serum-free medium with a 50 ng/ml of the nerve growth factor (NGF) to promote the differentiation of the cells. The cells were cultured a further 2 days as the same previous condition and their neurite outgrowth properties on the modified surfaces were observed by phase contrast microscope (CK2, Olympus).

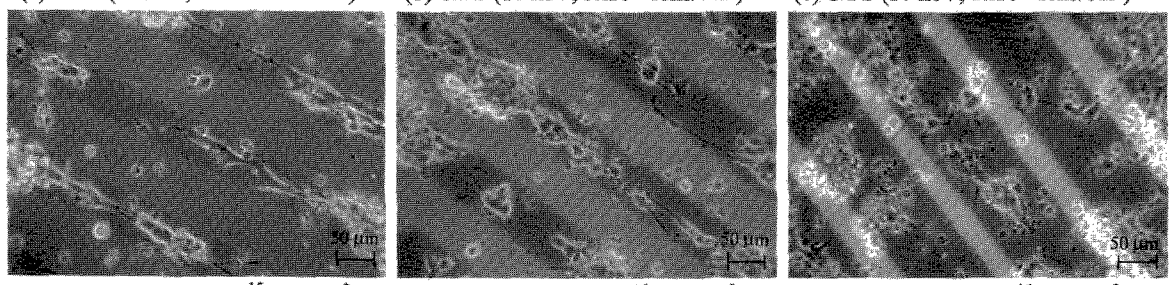
3. RESULTS AND DISCUSSION

3.1 Dose-implantation dependence of neurite outgrowth

Some phase contrast micrographs of PC12h cells cultured with NGF for 2 days on the C-implanted polystyrene films at 10 keV with $1x10^{14}$, $3x10^{14}$, $1x10^{15}$, $3x10^{15}$, $1x10^{16}$ and $3x10^{16}$ ions/cm^2 are shown in Fig. 1.



(a) C/PS (10 keV, $1x10^{14}$ ions/cm^2) (b) C/PS (10 keV, $3x10^{14}$ ions/cm^2) (c) C/PS (10 keV, $1x10^{15}$ ions/cm^2)



(d) C/PS (10 keV, $3x10^{15}$ ions/cm^2) (e) C/PS (10 keV, $1x10^{16}$ ions/cm^2) (f) C/PS (10 keV, $3x10^{16}$ ions/cm^2)

Fig. 1. Phase contrast micrograph of PC12h cells with their neurite after 2-day NGF-supplemented culture on the C-implanted films at 10 keV as a function of ion dose at: (a) $1x10^{14}$, (b) $3x10^{14}$, (c) $1x10^{15}$, (d) $3x10^{15}$, (e) $1x10^{16}$ and (f) $3x10^{16}$ ions/cm^2.

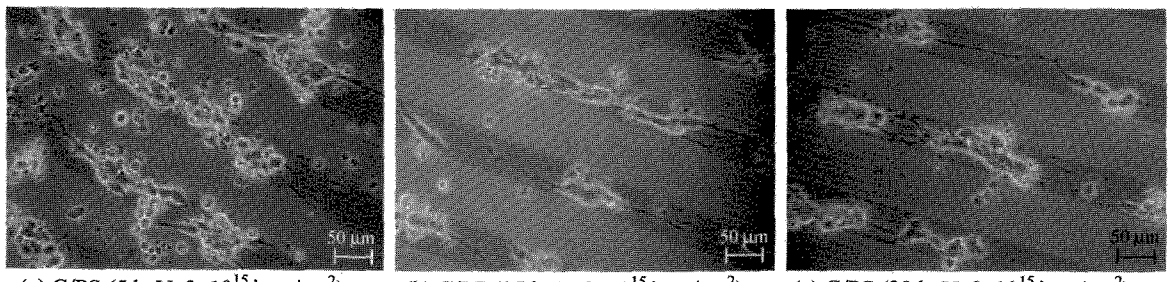
Figure 1 shows that the numbers and the length of neurite outgrowth increase as increase in the ion doses. At low dose such as $1x10^{14}$ and $3x10^{14}$ ions/cm^2 (Figs. 1(a) and 1(b)), small amount of cells with a group of floating cells weakly attached on the implanted surface, where is the narrow region between dotted lines, and a few cells differentiated their body and extended the long neurite length. Neurite outgrowth on the surface implanted at the medium dose such as $1x10^{15}$ and $3x10^{15}$ ions/cm^2 are shown in Figs. 1(c) and 1(d), respectively. The cells and their neurite outgrowth attached only on the implanted surface, where is the dark narrow stripe. No observation of abnormal cell bodies was found. However, at high dose as shown in Fig. 1(e) for $1x10^{16}$ ions/cm^2 and Fig. 1(f) for $3x10^{16}$ ions/cm^2, some cell bodies look different from that of PC12h cells on the reference dish (see Fig. 3(c)), and some cells with their extended neurite were observed on both implanted and unimplanted regions. As previous work, the optimum contact angle is obtained at $3x10^{15}$ ions/cm^2 [6], and from cell culture on all dose-implanted surfaces at 10 keV the best condition for neurite outgrowth of all energy implantation should be at this dose. All present results showed that the dose of about 3 ($x10^{15}$) ions/cm^2 for 5-20 keV is the best condition for the neurite-outgrowth property.

3.2 Energy-implantation dependence of neurite outgrowth

Phase contrast micrographs of the PC12h cells with their neurite outgrowth on the C-implanted films with $3x10^{15}$ ions/cm^2 as the function of energy at 5-20 keV are shown in Fig. 2.

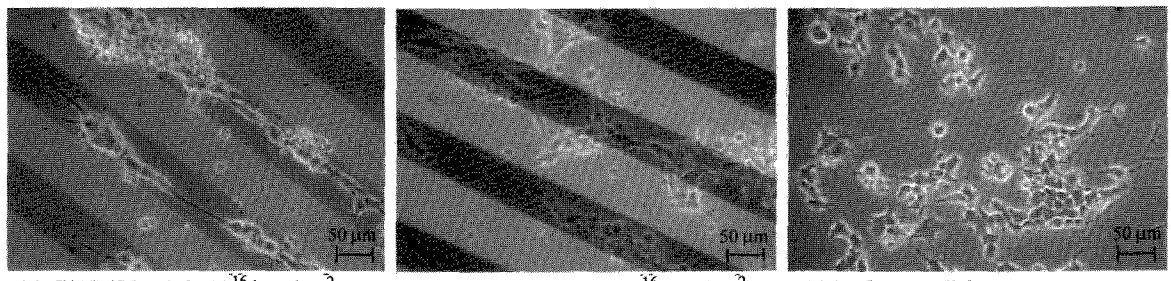
No significant differences in the neurite outgrowth on the C-implanted films with $3x10^{15}$ ions/cm^2 at 5-20 keV were seen. A lot of PC12h cells also differentiated and extended their neurite only on the implanted region. The neurite extension on the implanted region stopped at the edge of implanted region. No neurite on the unimplanted surface were found.

As the results of PC12h cells cultured on the polystyrene films implanted with 10 keV at low dose, merely number of cells still attached on the implanted surface after changed the culture medium before the NGF supplement. Unfortunately, at the same low dose with other energies after changed the medium, all cells detached from the surface because of their weakly attachment. Then, there is no result from the neurite outgrowth of PC12h cells on the C-implanted polystyrene at low dose such as $1x10^{14}$ and $3x10^{14}$ ions/cm^2.



(a) C/PS (5 keV, $3x10^{15}$ ions/cm^2) (b) C/PS (15 keV, $3x10^{15}$ ions/cm^2) (c) C/PS (20 keV, $3x10^{15}$ ions/cm^2)

Fig. 2. Phase contrast micrograph of PC12h cells with their neurite after 2-day NGF-supplemented culture on the C-implanted films at $3x10^{15}$ ions/cm^2 as a function of ion energy at: (a) 5, (b) 15, and (c) 20 keV.



(a) C/PS (5 keV, $3x10^{16}$ ions/cm^2) (b) C/PS (20 keV, $3x10^{16}$ ions/cm^2) (c) Reference dish

Fig. 3. Phase contrast micrograph of PC12h cells with their neurite after 2-day NGF-supplemented culture on the C-implanted films at high dose $3x10^{16}$ ions/cm^2 as a function of ion energy at: (a) 5, (b) 20 keV and (c) on the reference dish.

While culture results of PC12h cells on the polystyrene films implanted with 10 keV at high dose such as $1x10^{16}$ and $3x10^{16}$ ions/cm^2 does not show the good results for neurite outgrowth. Also, at the same high dose implantation with other energies, a number of PC12h cells attached, differentiated and extended the neurite on the C-implanted region. However, some cells attached, differentiated and extended their neurite on the implanted regions. Moreover, some cell bodies look different from that of PC12h cells on the reference dish. The cells are abnormal in shape with a large size. The abnormal in area attachment and neurite outgrowth of PC12h cells on the C-implanted polystyrene films at $3x10^{16}$ ions/cm^2 with low and high energy such as 5 and 20 keV are shown in Figs. 3(a) and 3(b), respectively.

Attached cells with their neurite outgrowth on the unimplanted surface, as well as the abnormal cells increased with the energy of implantation.

3.3 Neurite length extension

The average and the maximum neurite length of PC12h cells cultured on the C-implanted polystyrene at various ion doses and energies are shown in Figs. 4 and 5.

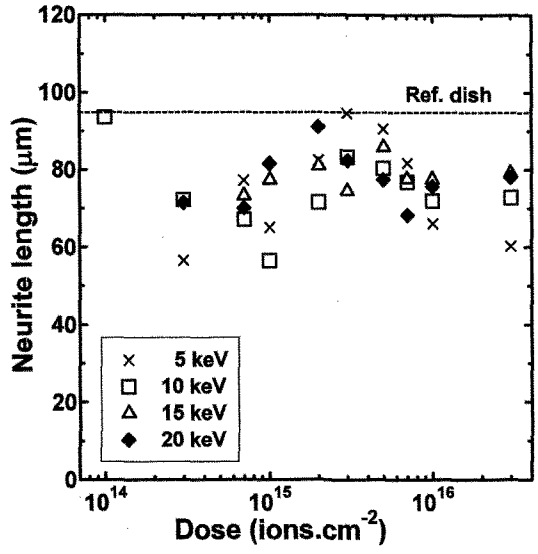


Fig. 4. The average neurite length of PC12h cells on the C-implanted polystyrene films at 5-20 keV as a function of ion dose.

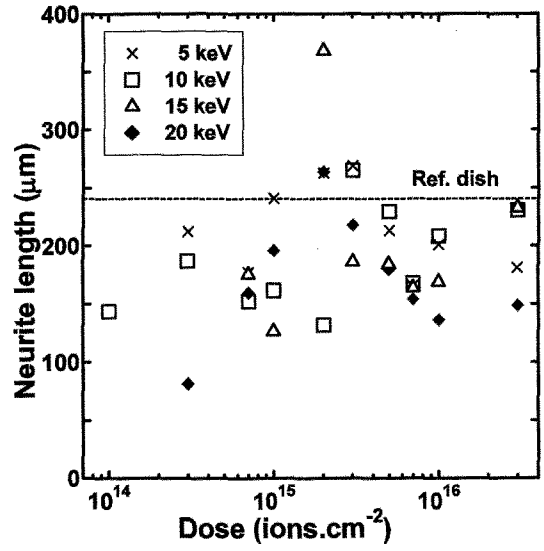


Fig. 5. The maximum neurite length of PC12h cells on the C-implanted polystyrene films at 5-20 keV as a function of ion dose.

After 2-day culture with NGF, the PC12h cells on the C-implanted films at 5-20 keV with 2-3 ($x10^{15}$) ions/cm^2 could extend very long neurite length as same as on the type-I collagen coated reference dish. Therefore, type-I collagen coating can be replaced by the surface of polystyrene films modified by carbon negative ion implantation at 2-3 ($x10^{15}$) ions/cm^2.

The observation of neurite outgrowth for each day showed that PC12h cells started to differentiate their cell bodies to extend the neurite after supplement NGF within 2 hours, so the average neurite-length at 0 day is about 10 to 20 μm as the same length as the cell body. After supplement NGF for 1 and 2 days, the longer neurite length is obtained. However, the average neurite-length growth rates decreased at the second day after supplement NGF and they may become saturate. Data are shown in Fig. 6. The average neurite-length growth rates for PC12h cells cultured on the polystyrene films implanted by $3x10^{15}$ ions/cm^2 at 5-20 keV are the same that corresponding to the nearly same contact angle at this dose. Moreover, they are similar to the rate of the cells cultured on type-I collagen-coated dish.

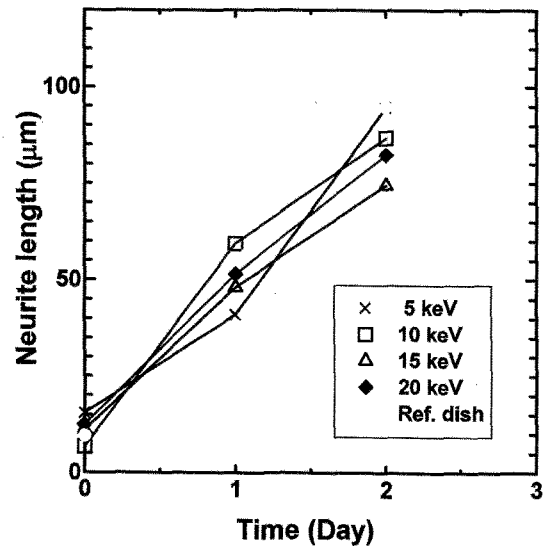


Fig. 6. The average neurite length for each day of PC12h cells on the C-implanted films at 5-20 keV with $3x10^{15}$ ions/cm^2 after supplement NGF.

The frequency distribution of neurite outgrowth of PC12h cells cultured in the serum-free medium containing 50 ng/ml of NGF for 2 days on the polystyrene films implanted at 10 keV and $3x10^{15}$ ions/cm^2 is shown in Fig. 7.

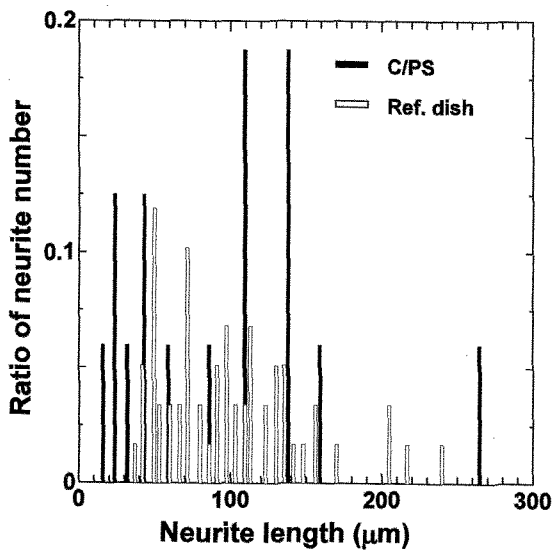


Fig. 7. Frequency distribution of neurite outgrowth on the C-implanted polystyrene films at 10 keV and $3x10^{15}$ ions/cm^2 and on a reference dish after 2-day NGF supplement.

PC12h cells have two methods for the increase in neurite length. One is the continuous length extension from the previous culture day and other is the new differentiation from the cell body. The obtained neurite from the first method has very long length with many branches on the main neurite, while that from the latter method has only the extended lengths with 10 to 15 μm from the cell body. From Fig. 7, ninety percents of frequency distribution of the average length are around 25-170 μm for both cells cultured on the C-implanted films and on the type-I collagen-coated dish. The highest distribution value for the C-implanted films is around 110 and 140 μm, while that for a reference dish is around 50 μm. It means that almost all neurite lengths on the C-implanted polystyrene films at 10 keV with $3x10^{15}$ ions/cm^2 increase by continuous length extension from the previous culture day. Only five percents of the distribution for the average neurite length are from the new differentiation from the cell body.

4. CONCLUSION

Neurite outgrowth property of PC12h cells cultured on the C-implanted polystyrene films is controlled by various the ion doses and energies. The suitable dose for the neurite outgrowth property is in the order of 10^{15} ions/cm^2. The best condition for that property is around 2-3 ($x10^{15}$) ions/cm^2. At this dose, the maximum value of the average neurite length of cells is equal to that of cells cultured on type-I collagen-coated dish. Then, type-I collagen coating can be replaced by carbon negative ion implantation at the best conditions. From the results of the average neurite growth rate and that of the frequency distribution of the average neurite length, almost all neurite on the C-implanted films increased by the continuous lengths extension from previous culture day.

5. REFERENCES

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