

SELECTIVE NEURITE OUTGROWTH ON SILVER NEGATIVE ION (Ag⁻)-IMPLANTED POLYSTYRENE SURFACES*

Hiroko Sato^{a**}, Hiroshi Tsuji^b, Hitoshi Sasaki^b, Shinichi Ikemura^b, Yasuhito Gotoh^b,
Junzo Ishikawa^b and Sei-ichi Nishimoto^c

Departments of ^aPolymer Chemistry, ^bElectroinic Science and Engineering, and ^cEnergy and Hydrocarbon Chemistry,
Graduate School of Engineering, Kyoto University, Katsura, Kyoto 615-8510, Japan

Abstract The negative ion implantation technique was applied to modify polymer surfaces of culture dishes for neuronal cells, PC12h. The silver negative ion (Ag⁻)-implantation was carried out at an ion energy of 20 keV and a dose of 3×10^{15} ions/cm² with non-treated polystyrene (NTPS), tissue culture polystyrene (TCPS), and collagen-coated TCPS-Iwaki (CCPS). Ag⁻-implanted surfaces of Ag/NTPS, Ag/TCPS, and Ag/CCPS were studied with respect to contact angle and/or chemical composition. The numerical values of contact angles on Ag/NTPS and Ag/TCPS were similar within experimental error, indicating the resemblance in their hydrophobicity and hydrophilicity. The PC12h cells, however, were attached only to the Ag⁻-implanted region of NTPS, but not to the non-implanted NTPS region. Moreover, the neurite outgrowth was also observed to extend specifically along the Ag⁻-implanted region of NTPS but not on the non-implanted NTPS region, although neurites extended towards all directions on collagen-coated TCPS as a control surface. There was no remarkable difference in neurite outgrowth among Ag⁻-implanted regions of TCPS and CCPS. Thus Ag/NTPS region was affirmed to promote highly selective attachment, growth, and differentiation of PC12h cells, although its mechanism is still unknown.

Keywords Ag⁻-implanted polystyrene, Contact angle, Collagen, Neuronal cells

INTRODUCTION

A negative ion-implantation technique^[1, 2] developed by us for use in semiconductors, integrated circuits (IC), and large scale-integrations (LSI) has been studied in its application on biotechnology. The implantation of high ion energy beams causes the impairment of insulators such as common synthetic polymers. Our negative ion-implantation, however, is considered to result in less charge-up problems compared with positive ion-implantation. That is, negative ionic species can be implanted at a lower energy than positive ionic species, indicating less heat generation to insulators.

As a result of silver-negative ion (Ag⁻)-implantation, the polystyrene (Ag/NTPS) surfaces were changed to hydrophilic, and showed good biocompatibility towards human umbilical vein endothelial cells (HUVEC)^[3]. As adhesive cells like vascular endothelial cells are unable to survive on NTPS, polystyrene surfaces specially treated for tissue culture (TCPS) are used for their cell culture. Vascular endothelial cells, however, were observed to grow on the Ag/NTPS region but not on the non-implanted NTPS region in a dish^[3]. In the case of neuronal cells, rat adrenal pheochromocytoma cells, PC12h were observed to grow specifically on negative ion-implanted regions of Ag⁻, Cu⁻, and C⁻^[4]. The cells are known to express neuronal properties to extend neurites such as dendrites and axons^[5]. Moreover, no prominent difference in cell growth among those negative ion species was observed^[4].

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**Corresponding author: Hiroko Sato (佐藤弘子), E-mail: hsato@polym.kyoto-u.ac.jp

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Recently, tissue regeneration and engineering has attracted attention as one of the therapies for repairing diseased organs, impaired tissues, as well as wounds. The proliferation and differentiation of stem cells were studied extensively in relation to cytokines and cell matrix materials and architecture of both natural and synthetic origins. Nerve cells extend their dendrites, axons, neurotubules, and neurofibrils *in vivo* along basement membrane mainly composed of laminin, collagen and fibronectin. In this work, effects of Ag⁻-implanted artificial surfaces and collagen will be studied on the biocompatibility of growth and differentiation for PC12h cells.

EXPERIMENTAL

Sample Dishes for Ag⁻-Implantation

Plastic dishes (6 cm in diameter) for cell culture of NTPS (No. 25060-60, Corning), TCPS (No. 25010-60, Corning), and collagen-coated TCPS-Iwaki (CCPS, No. 4010-010, Iwaki) were used as samples for Ag⁻-implantation. Type I collagen (Böttger) derived from calf skin was coated on Ag/NTPS and TCPS as a control at a concentration of 0.04 mg/mL. 1 mL of the collagen solution in phosphate buffered saline (PBS) was used for coating on sample dishes for 45 min at 37°C. Then sample dishes were rinsed gently with PBS, and used for cell culture.

Apparatus for Ag⁻-Implantation

Figure 1 shows a schematic illustration of the apparatus for negative ion implantation. The negative ions were produced in a cesium-sputter-type negative ion source, *i.e.*, a neutral and ionized alkali metal bombardment-type negative ion source (NIABNIS)^[1, 2] with a sputtering target of highly pure silver (99.99%). The negative ion beam was transported to a Ag⁻-implantation chamber known as a Faraday cup through a limiter formed with a hole of 11.28 mm in diameter after mass separation with an electromagnetic sector at a bending angle of 30°. A pattern for Ag⁻-implantation was designed on a mask placed over the center of sample dishes. The mask made of nickel had 67 slits and 66 spacings of size 4 × 8 mm and thickness 40 μm. The slits formed a pattern of 67 stripes one of which was 60 μm wide and 4 mm long on the center of sample dishes by negative ion implantation. Those dark stained stripes are shown in Figs. 2(a) and 2(b). Sample dishes were placed on the collector cup in the implantation chamber and implanted with Ag⁻ possessing an ion energy of 20 keV at a dose of 3 × 10¹⁵ ions/cm² and current density less than 200 nA/cm². Residual and background gas pressures were approximately 1.0 × 10⁻³ Pa and 1.4 × 10⁻⁴ Pa, respectively.

Characterization of Ag⁻-Implanted Surfaces

Negative ion implantation should cause chemical degradation of the surface vicinity of polymer dishes. The implanted negative ions of Ag⁻ should lose electron and be neutralized to Ag atoms in the vicinity of material surfaces. The surfaces of Ag⁻-implanted sample dishes were characterized by the measurement of contact angle against highly deionized water and X-ray photoelectron spectroscopy (XPS). The XPS spectra were obtained using an AXIS-165S (Shimadzu/Kratos, Kyoto) apparatus with monochromatic AlKα as the X-ray source. The XPS spectra of Ag/NTPS and Ag/TCPS were obtained, and the C_{1s} spectra were analyzed with a computer equipped with the XPS apparatus. The content of (1) aliphatic and aromatic carbons (C—C and C—H), (2) ether carbon (C—O), (3) carbonyl carbon (C=O), and (4) ether and carbonyl carbon (O—C=O) was estimated from the analysis of the C_{1s} spectra.

The hydrophilic and hydrophobic properties at sample surfaces were investigated from the measurement of contact angle against deionized water within 2 h after Ag⁻-implantation into samples. That is, pictures of water droplets (0.8–1.2 μL) on the sample surfaces were taken from a horizontal direction at approximately 20°C. The numerical contact angle values were calculated from the width and height of a droplet assuming a circular shape. Seven values obtained from pictures of droplets were averaged for each sample.

Cell Culture

The Ag⁻-implanted samples were sterilized in 70% aqueous ethyl alcohol solution and allowed to stand overnight, rinsed and immersed three times in sterilized and deionized water for 2 days before cell culture. Then

the dishes were dried for 1 day before they were used for cell culture or collagen coating, although Ag/CCPS dishes were not sterilized. The cells of approximately 10^5 cells/cm², one twentieth of the cell density in the confluent state were plated to each dish, and cultured for 2 days.

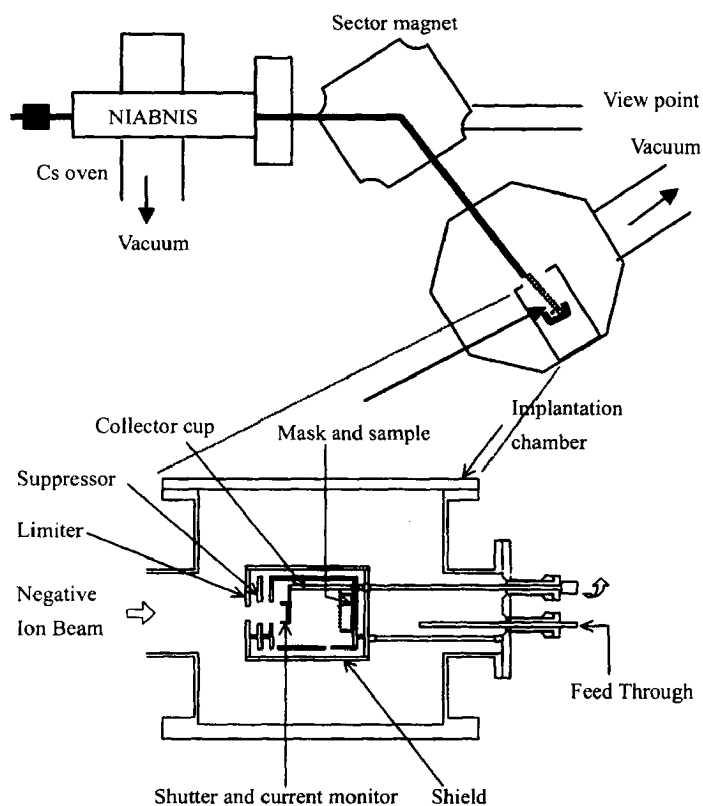


Fig. 1 Schematic illustration of an apparatus for negative ion-implantation and a Faraday box attached with a sample

If necessary, a mask (broken line) was placed over a sample.

Rat adrenal pheochromocytoma cells (PC12h) were cultured in a Dulbecco's modified Eagle medium (DMEM, Nissui, Tokyo) supplemented with 5% fetal bovine serum (FBS, Biowhittaker Products, Walkersville), 5% horse serum (HS, Sera-Lab, Crawley), potassium benzyl penicillin (50 units/mL, Meiji Seika, Tokyo), and streptomycin sulfate (100 µg/mL, Meiji Seika) under 5% CO₂ at 37°C.

For the purpose of differentiating PC12h cells, the culture medium was changed to serum-free DMEM containing antibiotics described above. Then murine nerve growth factor (NGF, 2.5S, Upstate Biotechnology, Lake Placid) was added at a concentration of 50 ng/mL in serum-free supernatants of cell culture. The neurite outgrowth of PC12h cells was observed under a phase-contrast microscope (Olympus, Type CK-2, Tokyo) after for 2 days.

The growth of PC12h cells was observed from the cell density in a given area (0.0432 mm²) under the microscope. The length of neurites in an area of 0.0372 mm² was measured by using image analysis softwear Winroof (Mitani).

Assay for Nitrite Concentrations in Culture Medium

In the process of differentiation such as neurite outgrowth, PC12 cells have been reported to produce nitric oxide^[6], possibly oxidized to nitrite (NO₂⁻) and nitrate ions in culture supernatants. In order to reduce the possible oxidization of nitrite ions in culture supernatants the cells were cultured for 1 day and subsequently differentiated by NGF in serum-free medium for one additional day. Nitrite ions reacted with 2,3-

diaminonaphthalene (DAN, Dojindo, Kumamoto) to form 1-naphthotriazole, a fluorescent substance^[7-9]. DAN (25 μ L, 7.6 μ mol/L) in 0.62 mol/L HCl was added to a mixture (1 mL) containing one fifth culture supernatants diluted with deionized water for 10 min, and NaOH (12.5 mL, 2.8 mol/L) was added to the mixture. The concentration of 1-naphthotriazole formed^[6] was determined by using a fluorescence spectrometer (F-2000, Hitachi, Tokyo) at 450 nm upon excitation at 365 nm. Calibration curve of fluorescence intensity was linear in the range of 0.1 to 10 μ mol/L NO₂⁻.

RESULTS AND DISCUSSION

Ag⁻-Implanted Surfaces

The Ag⁻-implanted polymer surfaces were stained dark brown under our implantation conditions (Figs. 2a and 2b), suggesting the occurrence of degradation and carbonization of polymer surfaces. The composition and contact angle of Ag⁻-implanted surfaces are shown in Table 1. The contact angle (86°) of NTPS was decreased by Ag⁻-implantation at an ion energy of 20 keV and an ion dose of 3 × 10¹⁵ ions/cm², while the contact angle (66°) of TCPS was increased by Ag⁻-implantation. The contact angle of Ag/NTPS was almost the same as that of Ag/TCPS within experimental error, although the contact angle of Ag/CCPS was somewhat higher than that of Ag/NTPS and Ag/TCPC.

The content of C—C bonding and oxidized carbon forms were estimated from XPS C_{1s} spectra (Table 1). The atomic content of hydrogen could not be measured by XPS because of its small molecular weight. The silver content of ca. 10²⁰ to 10¹⁸ atoms/cm³ was detected in the surface vicinity from the surface to 200 nm in depth by secondary ion mass spectrometry (SIMS)^[3]. Its content was too small in the surface vicinity of ca. 5 nm in depth to be detected by XPS.

As a result of Ag⁻-implantation under our implantation conditions, the oxidization of polymer surfaces should occur definitely. That is, the contents of oxidative forms such as C—O, C=O, and O—C=O groups increased at the surface of NTPS. Moreover, the content of C=O and O—C=O groups at Ag/NTPS surface may be similar to Ag/TCPS surface despite the slightly lower content of C—O groups. Thus, their hydrophobic and hydrophilic properties are considered to be similar between Ag/NTPS and Ag/TCPS. The contact angle of CCPS was estimated to be 50 to 60° (not listed on Table 1). Therefore, both Ag/TCPS and Ag/CCPS surfaces are more hydrophobic than TCPS and CCPS, probably owing to the loss of hydrophilic groups.

Table 1. Composition, as analyzed with XPS C_{1s} spectra, and contact angle at Ag⁻-implanted surfaces

Surfaces	Composition (%)				Sum of oxidative form	Contact angle (deviation)
	C—C	C—O	C=O	O—C=O		
NTPS	100				0	
Ag/NTPS	79.54	13.07	5.68	1.70	20.45	86.2
Ag/TCPS	77.81	14.44	5.69	2.06	22.19	75.8
						(-1.0, +1.2)
Ag/CCPS						77.3
						(-3.0, +0.6)
TCPS						81.8
						(-2.1, +3.5)
						66.0

Cell Population and Neurite Outgrowth

The cell population on Ag⁻-implanted polymer dishes was investigated on three regions, (1) the Ag⁻-implanted region, (2) the peripheral region contiguous to Ag⁻-implanted region, and (3) the region distant away from Ag⁻-implanted region. The ratio of three areas on dishes corresponds to 1.0:1.0:98.0. The population of PC12h cells cultured for 2 days in the medium and subsequently for another 2 days in the serum-free medium in the presence of NGF is shown in Table 2. The remarkably high cell density was observed for the Ag/NTPS with and

without collagen coating.

The cells seemed to escape toward Ag/NTPS and the contiguous regions from the unimplanted NTPS region where no adhesive neuronal cells can survive. The cell population on Ag/TCPS was remarkably lower than that on TCPS and collagen-coated TCPS of the control surface, indicating that PC12h cells settled more preferably on TCPS than on Ag/TCPS. The same results were observed in the case of vascular endothelial cells on Ag/NTPS^[10]. The cell population on three regions of a Ag/CCPS dish was almost similar to that of the control. Thus, Ag/NTPS surface exhibited excellent selectivity to attach neuronal cells.

Table 2. Cell population of PC12h cells cultured for 2 days and differentiated by NGF for a further 2 days on Ag⁻-implanted material surfaces. Field corresponds to 0.0432 mm²

Material surfaces	Cell population, cells/field		
	Ag ⁻ -implanted region	Region contiguous to Ag ⁻ -implanted	Region distant from Ag ⁻ -implanted
Ag/NTPS	748.0 ± 91.6	99.5 ± 55.4	0
Collagen-coated Ag/NTPS	864.0 ± 270.1	12.8 ± 24.1	15.0 ± 22.7
Ag/TCPS	52.3 ± 27.3	189.0 ± 58.1	121.5 ± 42.2
Ag/CCPS	139.8 ± 66.4	204.8 ± 21.3	153.8 ± 46.5
Collagen-coated TCPS: Control	166.0 ± 13.0		

Effects of Collagen on Ag⁻-Implanted Polymer Surfaces

The differentiation of PC12h cells was studied by adding NGF into the serum-free medium. The cells extended relatively long neurites on Ag/NTPS and collagen-coated Ag/NTPS as compared with the control (Figs. 2 and 3). The neurite outgrowth on Ag/NTPS was observed to extend specifically along the Ag⁻-implanted region (Figs. 2a and 3), while the cells extended their neurites in all directions on collagen-coated both Ag/NTPS (data not shown) and TCPS of the control (Fig. 2c).

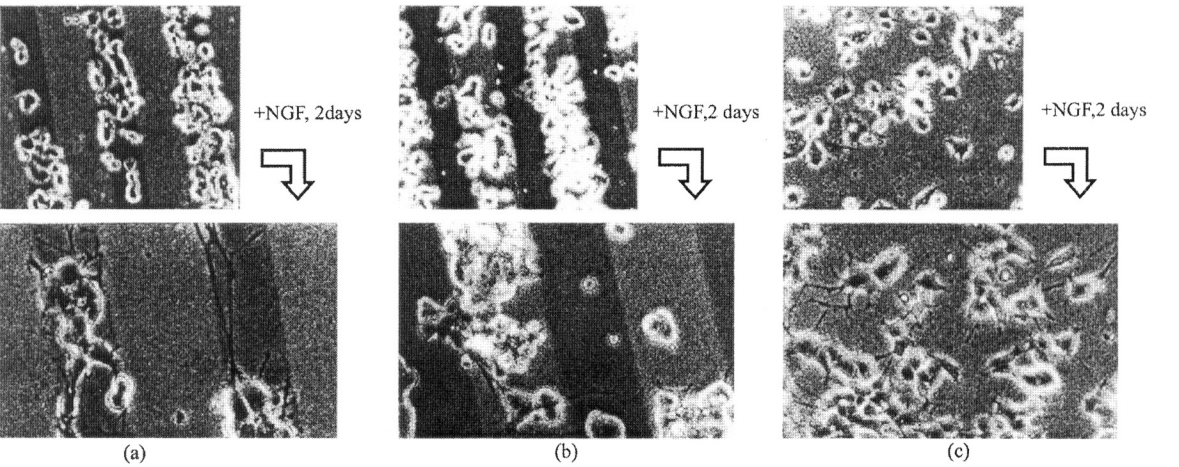


Fig. 2 Phase-contrast micrographs for PC12h cells cultured for 2 days, and differentiated by adding NGF in serum-free medium for another 2 days on Ag/NTPS (a), Ag/TCPS (b), and collagen-coated TCPS (c) as control. One repeating stripe unit corresponds to 120 μm. Dark stripe portions correspond to the Ag⁻-implanted region.

The frequency in length of all neurites extended on Ag/CCPS and CCPS (data not shown) was lower than that of the control. It may be related to some denaturation of collagen molecules on CCCP. Moreover, the amino acid residues for the cell adhesion on collagen molecules coated on Ag/CCPS may be degraded by the negative

ion implantation. On collagen-coated Ag/NTPS, the cells grew better than just on Ag/NTPS (Table 2). However, collagen-coated matrix reduces the specificity to cell attachment (Fig. 2) and neurite outgrowth. A high selectivity of cell attachment and neurite outgrowth limited on Ag/NTPS surface should be attributed to Ag⁻-implantation, although the effect of Ag atoms on neurites is not clear yet.

Nitrite Concentration in Serum-free Culture Medium Related to NO Production

PC12h cells were reported to produce nitric oxide (NO) by neural NO synthase (nNOS) as a result of NGF-induced differentiation^[6]. Nitric oxide produced could feasibly be oxidized to NO₂⁻ and then to NO₃⁻ forms. The cells cultured in serum-free culture medium in the presence of NGF for 1 day gave increased nitrite concentrations with increasing cell density (Fig. 4). However, the ratios of nitrite concentrations in supernatants in the presence of the cells against DMEM in the absence of the cells resulted in 10.07 ± 1.67 for Ag/NTPS, 11.01 ± 0.04 for Ag/TCPS, 10.98 ± 0.27 for Ag/CCPS, and 11.54 ± 0.39 for collagen-coated TCPS, the control. Thus, the numerals indicate almost the same nitrite concentrations within experimental errors among media cultured on various dish surfaces. All PC12h cells did not participate in differentiation (Fig. 2), and therefore no clear difference in nitrite concentrations may be detected even on a stressful cell matrices such as Ag/NTPS^[3].

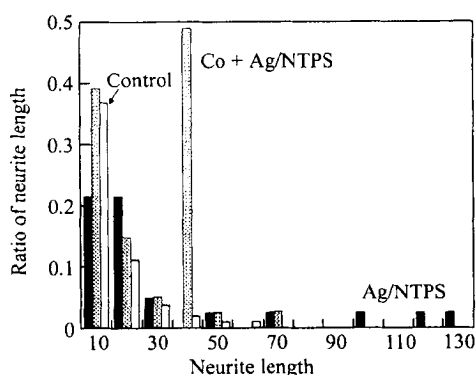


Fig. 3 Frequency distribution for neurite length of PC12 cells cultured and differentiated on artificial surfaces of Ag/NTPS (solid), collagen (Co)-coated Ag/NTPS (stippled) and collagen-coated TCPS as control (open)

The abscissa starts from 10 μm , approximate length of a cell body.

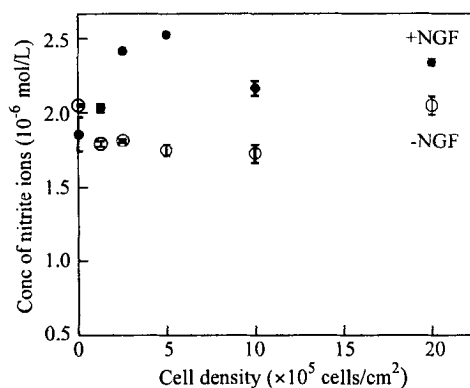


Fig. 4 Increase in nitrite concentration of supernatants related to NO production in neuronal differentiation of PC12h cells by NGF

Nerve Guidance

The surface modification may be achieved by physical methods^[11,12] such as negative ion implantation, plasma discharge, ion beam implantation and radiation as well as other physico-chemical methods^[13]. The outgrowth and extension of neurites were reported to be induced from extracellular matrices^[14-16] which were composed of natural and artificial materials. Neurite outgrowth of mouse dorsal ganglion cells (DRG) increased in grooves from 0.1 to 10 μm in depth on quartz glass^[11]. The guiding effects were investigated on conductive polymer (oxidized polypyrrole) for PC12 cells^[12]. Moreover, mouse and chick DRG extended their neurites along metal oxide patterns whose guiding effects were dependent on the electronegativity of the metal on silica glass^[12]. In the case of naturally derived matrix, neurites parallel to some direction of embryonic chick DRG were observed to extend on fresh-frozen sections of rat sciatic nerve^[14].

On the border of our Ag⁻-implanted and non-implanted NTPS regions, no clear grooves or morphological changes were observed under an atomic force microscope^[17]. The roughness between Ag/NTPS and non-implanted regions was estimated to be approximately 1.5 nm. Such roughness indicates no remarkable change at

surfaces produced by Ag⁻-implantation. In addition, it looks too small as compared with 10 µm of the average size of cell body to recognize as grooves. Therefore, selective effects of Ag/NTPS as nerve guidance are considered to result from protein adsorption suitable for the attachment of neuronal cells. For example, cell adhesive proteins such as laminin, collagen, fibronectin and neuronal cell adhesion molecule (NCAM)^[15] and even their fragments contained in the medium may contribute to the mechanism for high selectivity. Cell adhesive motif of RGDS (Arg-Gly-Asp-Ser) on protein fragments appears to be concentrated on the Ag⁻-implanted region, almost one hundred area of a NTPS dish, different from TCPS and CCPS. Such neuronal adhesion proteins concentrated on Ag/NTPS are considered to play a role in producing high populations of the cells and adhesion of growth cones, resulting in selective guidance of neurites^[16].

CONCLUSION

Silver negative ions were implanted into various artificial surfaces, among which non-treated polystyrene implanted at an ion energy of 20 keV and a dose of 3×10^{15} ions/cm² may be expected as an excellent nerve guidance. The Ag⁻-implanted surface (Ag/NTPS) was oxidized and became somehow hydrophilic from the measurement of XPS and contact angle. The Ag/NTPS surface brought about noticeable changes to neuronal cells, PC12h by Ag⁻-implantation. That is, high selectivity was observed for cell attachment and differentiation of neurite outgrowth. Probably, such a high specificity of the surface for neuronal cells was related to protein adsorption for cell attachment. Negative ion implanted polymers are expected to serve as artificial cell matrices for nerve regeneration.

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