Transactions of the Materials Research Society of Japan 29 [2] 575-580 (2004)

Improvement of Nerve-Cell Affinity of Silicone Rubber by Carbon-Negative-Ion Implantation and Its Application to Rat Sciatic Nerve Regeneration

Hiroshi Tsuji[1], Masayoshi Izukawa[1], Yoshiyuki Utagawa[1], Ryosuke Ikeguchi[2], Ryosuke Kakinoki[2], Hiroko Sato[3], Yasuhito Gotoh[1] and Junzo Ishikawa[1]

[1] Department of Electronic Science and Engineering, Kyoto University, Kyotodaigaku Katsura 1, Nishigyo-ku, Kyoto 615-8510, Japan, Phone: +81-75-383-2284, Fax: +81-75-383-2283, E-mail: tsuji@kuee.kyoto-u.ac.jp
[2] Department of Orthopedic Surgery, Kyoto University, Shogoin, Sakyo-ku, Kyoto 606-8501, Japan
[3] Department of Polymer Chemistry, Kyoto University, Kyotodaigaku Katsura 1, Nishigyo-ku, Kyoto 615-8510, Japan

Improvement of nerve-cell affinity of silicone rubber surface is expected for the nerve regeneration by using silicone tube in "chamber method". For surface modification by negative ion implantation, we investigated on contact angle and attachment properties of extracellular matrix as well as of nerve cell *in vitro*. And also *in vivo* nerve regeneration was tested. Carbon negative ions were implanted in a silicone rubber sheet at energy in 5 - 30 keV with a dose in 10^{14} - 10^{16} ions/cm^2. The contact angle decreased to 83° as increase in ion energy and in dose for C-implantation and it almost saturated over 3 x 10^{15} ions/cm^2. The decrease of contact angle is due to oxygen atoms adsorbed at induced defects. It was found that fibronectin and nerve cells, both attached on the C-implanted surface rather than on the original surface. In the regeneration test *in vivo* of rat sciatic nerve by using a C-implanted (10 keV, 3 x 10^{15} ions/cm^2) silicone tube (inner diam.:2 mm, outer diam.:3 mm), the sciatic nerve was regenerated between stumps with a distance of 15 mm in the C-implanted tube. Twenty-four weeks after the "tubulation" the sciatic nerve was also functionally repaired so that the electric impulse was transferred through the regenerated region. This result is superior to that of unimplanted silicone tube.
Key words: Negative ion implantation, Cell attachment, Silicon rubber, Nerve regeneration, Rat sciatic nerve.

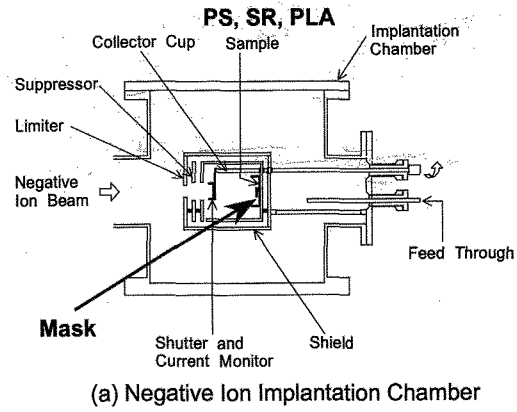
1. INTRODUCTION

Surface treatment by ion implantation of polymer surface with attempts to use in biological and medical field has been progressed and remarkable results have been reported about improving biocompatibility and cellular affinity.. Not only vascular cells, but also nerve cells were selectively attached on the ion implanted polymer surfaces. In the nerve cell, adhesion property of nerve protrusion was also controlled by negative-ion implantation [1-4]. Recently, we investigated the improvement of adsorption property of proteins by the negative-ion implantation to the surface. In this paper, we described the fundamental properties of implanted polymer surfaces and an application of surface treatment by negative-ion implantation to guide tube of silicone rubber for nerve regeneration in a grafting operation of "tubulation" to rat sciatic nerve.[5, 6]

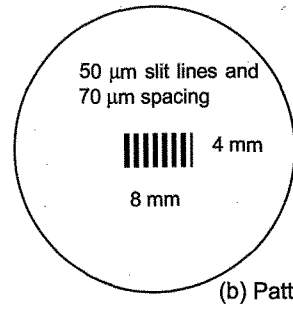
2. IMPROVEMENT OF ADHESION PROPERTIES OF NERVE CELL AND NEURITES

The negative-ion implantation has an advantage of "charge-up free" [7, 8] for insulators. Therefore, negative-ion implantation technique is considered to be very suitable for surface modification of polymers to improve attachment properties of nerve cell and neurite.

Mass-separated negative ions such as silver, carbon and copper were implanted to 5 surfaces of four kinds of polymers through a micro-patterning mask with many aperture of slit with 50 μm in width and 70 μm spacing in a implantation chamber of the negative ion implanter NIABNIS [9]. The current density during implantation was less than 400 nA/cm^2 and residual gas pressure less than 1 x 10^{-3} Pa. The implantation chamber and pattering mask were shown in Fig. 1. The typical implantation conditions were 10 keV(C), 20 keV(Ag) of implantation



(a) Negative Ion Implantation Chamber



(b) Patterning Mask

Figure 1. Schematic diagrams of (a) the negative-ion implantation chamber of NIABNIS and (b) the pattering mask.

energy and 3 x 10^{15} ions/cm^2 of dose. The five polymer surfaces were inner surface of a commercial available polystyrene dish PS (NTPS) (non-treatment polystyrene dish, Daw Corning Co. Ltd.), spin-coated polystyrene SCPS (5% polystyrene and toluene) on cover glass, a poly L-lactic acid sheet PLA (Ecoloju, Mitsubishi Jushi Co. Ltd.) and silicone rubber of medical use grade (Kameka Medics and Fuji systems). After sterilized the ion-implanted samples with 70 % ethanol and rinsed with pure water, nerve cells of PC-12h (rat adrenal pheochromocytoma) were seeded on the sample surfaces. The first 2 days the cells were cultured with Dulbecco's Modified Eagle's Medium (9.5g/l, DMEM. Nissui) containing 5% fatal bovine serum (FCS, Whittker Bioproduct), 5% heat-inactivated horse serum (FS, Gibo), 10% $NaHCO_3$ (37mg/l) and antibiotics in a humid incubator at 37 °C with 5 % CO_2 air flow. Then, the cells were continuously cultured in a serum-free DMEM added with nerve growth factor NGF (50 ng/ml) in the incubator for 2 days.

Attachment properties of nerve cell on various ion-implanted samples are shown in Fig 2, where phase

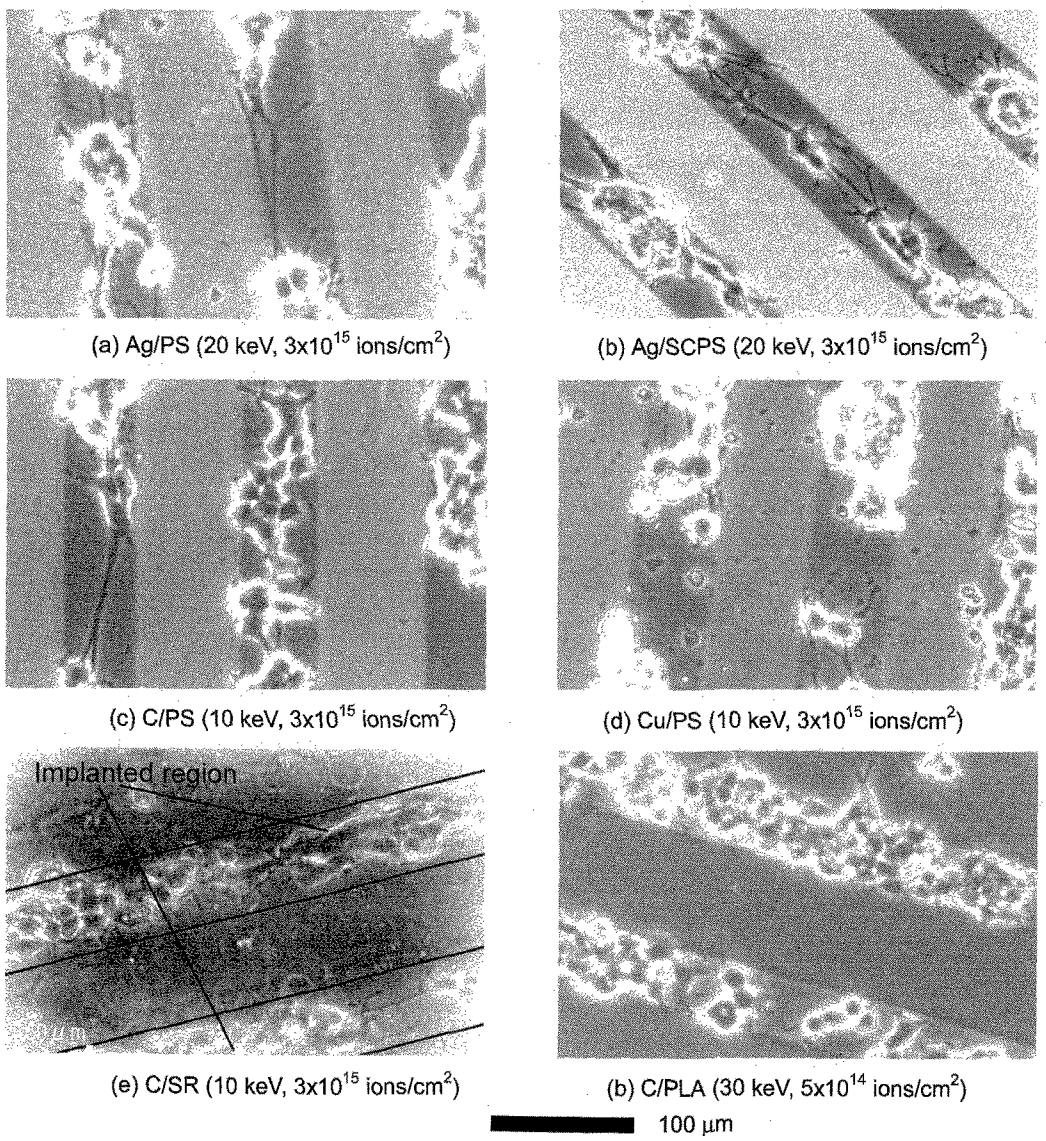


Figure 2. Phase contrast micrographs of PC-12h nerve cells cultured on the negative-ion implanted polymer surfaces for 4 days: (a) Ag-implanted polystyrene dish (Ag/PS), (b) Ag-implanted spin-coated polystyrene (Ag/SCPS), (c) C-implanted polystyrene dish (C/PS), (d) Cu-implanted polystyrene dish (Cu/PS), (e) C-implanted silicone rubber (C/SR), and (f) C-implanted poly L-lactic acid (C/PLA).

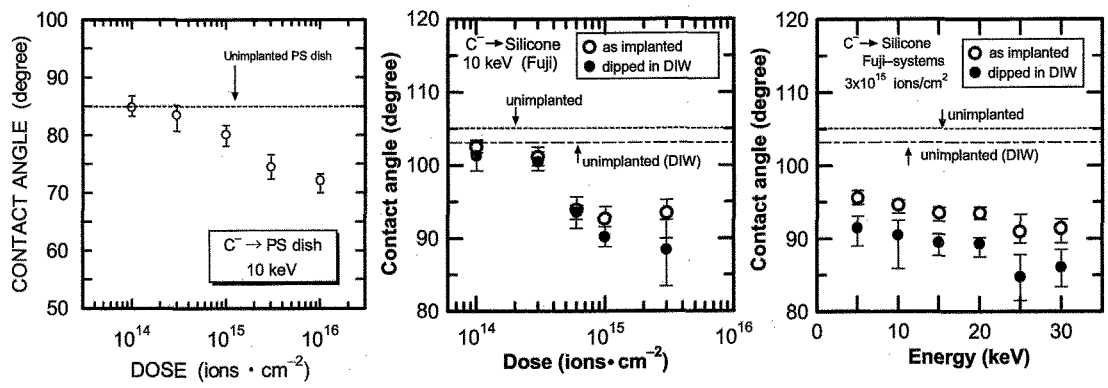


Figure 3. Contact angles of water (deionized water, DIW) for carbon negative-ion implanted (a) polystyrene dish and (b) silicone rubber at 10 keV with various doses, where open circles are shown the contact angle values measured just after the implantation within 2 hours and solid circles are the value measured after dipped in water for 2 hours.

contrast micrographs of nerve cells of PC-12h cultured in vitro on the sample surfaces. The nerve cells were attached only on the negative-ion implanted region and all neurites outgrown from cell body also extended over the implanted area.

3. WETTABILITY OF IMPLANTED SURFACES

Wettability of the surface in general is considered to strongly relate to the cell affinity. The wettability was evaluated by contact angle of deionized water. Figure 3 shows the change of contact angle for the modified (a) polystyrene PS and (b) silicone rubber SR (Fuji systems) surface implanted with carbon negative ions. In Fig. 3 (b) and (c), the solid circles shows the contact angle measured after dipping in water for 2 hours. In the dose dependence of contact angle at 10 keV, the contact angle decreased with an increase in dose. For C/PS at $3x10^{15}$ ions/cm^2, the angle decreased to 73 degrees from about 85 degrees. For C/SR, the contact angle decreased to 93 degrees as implanted and more decreased to 88 degrees after dipping in water from 105 degrees of unimplanted SR. The silicone rubber showed the decrease. The contact angle was almost saturated over the 3 x 10^{15} ions/cm^2 for SR. The energy dependence of contact angle was shown in Fig. 3 (c) for SR. Ag negative ion implantation also made the contact angle to decrease.

Thus, the surface of polymers was modified to become more hydrophilic by the negative-ion implantation.

4. SURFACE ATOMIC BONDING CHANGE

Atomic composition change of the polymer surface was investigated by X-ray photoelectron spectroscopy (XPS) between before and after the implantation. For the polystyrene, we confirmed oxygen atoms were introduced into the implanted surface. Also, the fraction of oxygen atoms in the surface of silicone rubber increased after the implantation. This increase in oxygen atoms means that adsorption of oxygen atoms from residual gas to ion-induced defects of the surface. Figure 4 shows the details of C 1s spectra for SR: (a) as received and (b) after carbon negative implantation at 10 keV with $3x10^{15}$ ions/cm^2. The broadening of C 1s spectra was observed in the higher region of bonding energy and means that the bonds of C-O(H) and C=O were resulted. The following claims are expected from

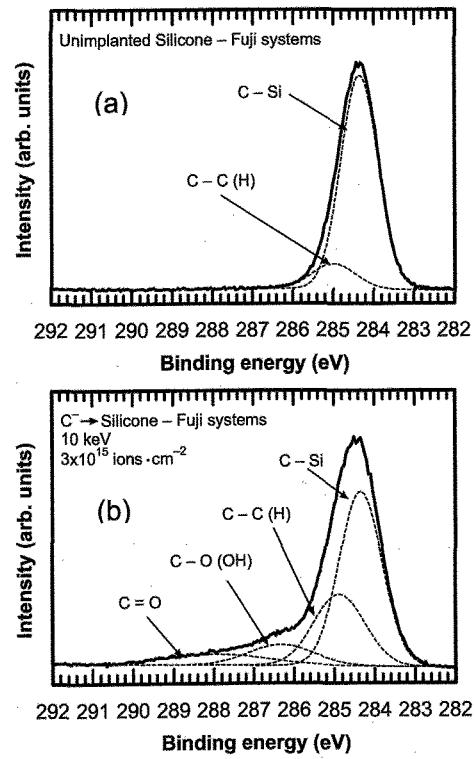


Figure 4. XPS C1s spectra of silicone rubber, (a) as received and (b) after carbon negative ion implantation.

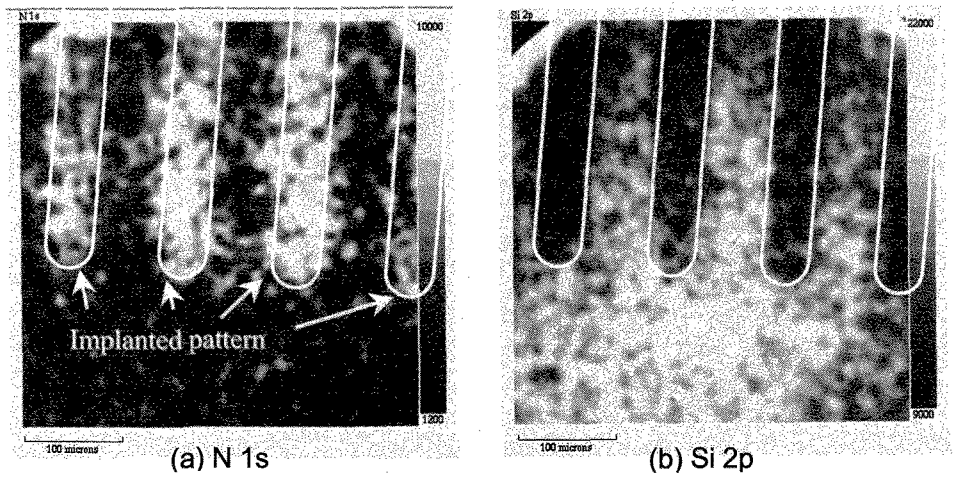


Figure 5. XPS element mapping of (a) N 1s and (b) Si 2p on the C-implanted silicone rubber surface after dipping in a culture medium with serum for 2 hours.

results of oxygen adsorption and broadening C 1s spectra. (1) ion bombardment resulted in the destruction of C-H bond, in the sputtering of C atoms in SR and made many defects, (2) oxygen atoms were adsorbed on the surface from ambient, and (3) the oxygen formed functional groups such as hydroxyl of OH on the surface. Thus the surface obtains hydrophilic property by ion implantation.

5. ADSORPTION PROPERTIES OF PROTEIN

Proteins are closely related to the cell attachment oh the surface. We investigated the adhesion properties of proteins on the carbon implanted silicone rubber surface. After implantation of carbon negative ions into the silicone rubber SR at 10 keV and $3x10^{15}$ ions/cm^2 through the pattering mask, the sample was dipped in the DMEM containing 5% fetal bovine serum and 5% heat-inactivated horse serum in an incubator at 37 °C for the protein adsorption from serum. Then, the sample were lightly rinsed with pure water and dried. Proteins in general include nitrogen atoms in the peptide bond. Therefore, detection of nitrogen atoms on the surface means the protein adsorption.

Figure 5 shows the surface mapping of (a) nitrogen atom and (b) silicone with intensity of detected N 1s and Si 2p in the same area of 400 x 400 μm of the sample. In the figure, white lines indicate the carbon implanted patterns. N atoms were detected on the C implanted region much more than unimplanted area. On the centrally, Si atoms were detected on the unimplanted area. The results means the proteins adhered on the implanted surface. Figure 6 shows the surface mapping of N 1s for the sample dipped in a fibronectin solution. The similar result of the selective adsorption of nitrogen was obtained. The detected nitrogen atoms corresponded to the protein of fibronectin. Thus, the improvement of protein adsorption properties was obtained by negative

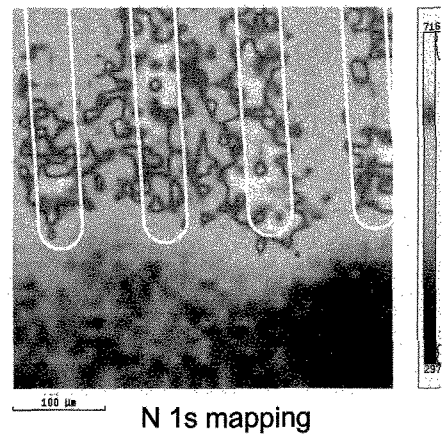


Figure 6. XPS N 1s mapping on the C-implanted silicone rubber surface after dipping in fibronectin solution. For 2 hours.

ion implantation. For the silicone rubber surface, the adsorption fraction of protein increased to 5 at.% from 1 at.% on the unimplanted SR.

6. SURFACE TREATMENT OF SILICONE RUBBER FOR "TUBULATION"

Silicone rubber (SR) has properties of stable and inactive in a living body and silicone rubber tube was investigated in many researches of nerve regeneration by means of "tubulation" [10-16]. Figure 7 shows an illustration of the "tubulation" (or "chamber method") for regeneration of the peripheral nerve system. The tubulation means a process for grafting operation for regeneration of nerve axon within an interneural stump gap through a tube of artificial material, and is expected to be an alternative method to autogenous nerve grafting. The recovered length of rat sciatic nerve was 10 mm at

(Received October 9, 2003; Accepted January 20, 2004)

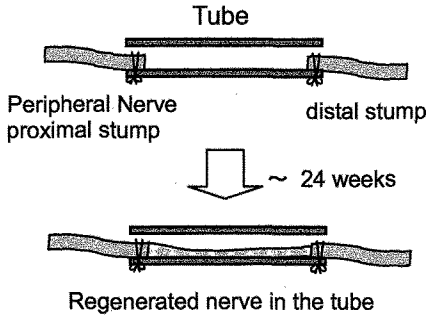


Figure 7. Illustration of "tubulation" (or "chamber method") for nerve regeneration.

maximum by using a silicone rubber tube. For long nerve regeneration, tubes containing various materials of laminin [11], dialyzed plasma [13], collagen [14] and blood vessels [15, 16] were investigated, and regeneration of rat sciatic nerve can be extended over an inter-stump gap of 15 mm. The longer distance of recovered nerve should be required for the actual surgical treatment. We considered that the nerve regeneration is strongly influenced by adhesive proteins for nerve-cell migration and also by induction factor proteins for axon extension and direction. We expected the longer nerve regeneration with increasing absorption of these proteins on the inner wall of the silicone rubber tube by ion beam modification. No direct modification of SR surface was reported. From the standpoint that contact guidance of nerve cell migration and axon extension from both nerve stumps plays an important role in nerve regeneration between the inter-stump gap, the inner surface of silicone rubber tube should be modified to have an improved neuron affinity.

6.1. Preparation of C-implanted Silicone Rubber Tube and "Tubulation" to Rat Sciatic Nerve System

For nerve regeneration test *in vivo*, 18-mm-long silicone rubber tube (SRT) was opened longitudinally and fixed on a polystyrene pedestal plate by sutures at both side edges to expose the inner surface to carbon negative-ion beams. After C-implantation at condition of 10 keV and $3x10^{15}$ ions/cm^2, the tube was released from the pedestal. It became again of tube shape and the slit was sealed with a small amount of liquid silicone rubber. Rats (Fisher 344, 10-12 weeks old) was used. All tubulation experiments were performed in accordance with the guidelines of the Animal Research Committee, Graduate School of Medicine, Kyoto University. The right sciatic nerve of rat was exposed and a nerve segment was removed in the middle thigh. Then, the edge of the proximal and distal nerve stumps was inserted about 1.5 mm into each side of the C-implanted silicone rubber tube (C/SRT) and sutured, leaving an inter-stump gap of 15 mm. Figures 8(a) and 8(b) show appearances before and after the grafting operation of "tubulation" with a C/SRT into rat sciatic nerve system. As controls, unimplanted silicone rubber tubes (SRTs)

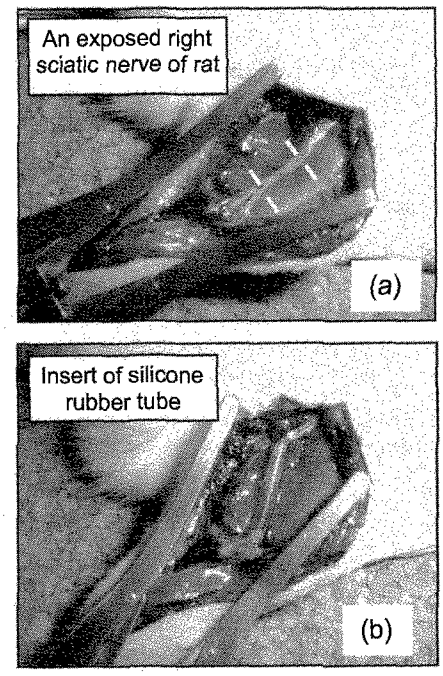


Figure 8. Photographs of the appearances (a) before and (b) after the grafting operation of "tubulation" with a C-implanted silicone rubber tube into rat sciatic nerve system.

were implanted as the same manner in the 5 rats. Electro-physiological property was investigated after 12 and 24 weeks since the tubulation.

6.2. Result of nerve regeneration in vivo by tubulation with C-implanted SR rubber tube [5, 6].

The rats were cut their right leg to be opened and the inserted C/SRT were exposed to check regeneration at 12 weeks and 24 weeks after the tubulation surgery. The neural tissue was developed between the inter-stump gap of 15 mm in the C-implanted silicone rubber tube (C/SRT) for both cases of 12 weeks and 24 weeks. Figure 9 shows the appearance of the neural tissue developed in the tube at 24 weeks. The number of the myelinated axons at the three sections of proximal, middle and distal parts of the regenerated nerve was counted for each cross-section and is listed in Table 1. The myelinated axons developed in the C/SRT were regenerated more than 50 % of the normal state of the left leg sciatic nerve. However, no neural tissue was developed in the unimplanted SRT at 24 weeks.

The stimulation transfer of electric pulse through the reconstructed nerve tissue was investigated No rat with C/SRT at 12 weeks showed any evoked action potentials in the pedal adductor muscle as well as rats with unimplanted SRT. At 24 weeks, the rats with C/SRT showed surely evoked action potentials to move the pedal adductor muscle. The mean motor nerve conduction velocity was 90.1 % of the value of the

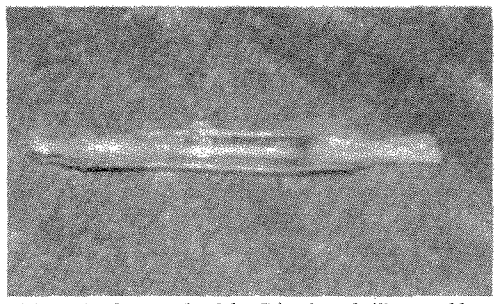


Figure 9. Photograph of the C-implanted silicone rubber tube at 24 weeks after the tubulation.. In the tube, a neural tissue was developed.

normal sciatic nerve system of left-hand leg of limb. And the amplitude of the action potential evoked in the pedal adductor muscle was 56.5 %.

Thus, the regeneration of the rat sciatic nerve within a inter-stump gap of 15 mm in length was obtained by the grafting operation of tubulation by using silicone rubber tube of which inner surface was modified by carbon negative-ion implantation. This long-distance recover of peripheral nerve system is considered to be due to the improved hydrophilic property, protein adsorption property and attachment properties of nerve cell and neurite by ion implantation. In nerve regeneration in a tube, the formation of fibrin matrix on the inner surface and replacement by a cellular, vascularized structure are important processes for axon growth and myelination [12]. The exudates emitted from the nerve stumps are considered to concern to the guidance of direction of axon growth. Therefore, the adhesion of exudates from the both nerve stumps into the tube is rapidly resulted with tight on a hydrophilic surface of C/SRT surface rather than unmodified SR.

7. CONCLUTION

The surface modification of polymers, in particular, silicone rubber by negative-ion implantation is shown. The ion implantation improved their wettability to bring better hydrophilicity, and better protein-adsorption property. This is due to the formation of functional groups such as hydroxyl. As a result, the attachment properties of nerve cell and neurite were improved. As an application of surface modification by the negative ion implantation, the "tubulation" of rat sciatic nerve by using carbon negative-ion implanted silicone rubber tube (C/SRT) was shown. The 15-mm-long sciatic nerve was found that the lacked nerve was regenerated at 12 weeks with more than 50 % myelinated axons in the C/SRT and that the nerve system was fully recovered of its function. .

ACKNOWLEDGMENT

We are grateful to the Nano-Structure Surface Evaluation Division of VBL, Kyoto University, for XPS measurement.

Table 1. Number of myelinated axon in regenerated nerve of the rat sciatic nerve system at 12 weeks and 24 weeks after the tubulation.

Part of Regenerated nerve	12 weeks	24 weeks
Proximal section	10973	11638
Middle section	8366	9070
Distal section	13276	11273

REFERENCES

[1] H. Tsuji, H. Satoh, S. Ikeda, Y. Gotoh, J. Ishikawa, Nucl. Instr. and Meth. B 141 (1998) 197.

[2] H. Sato, H. Tsuji, S. Ikeda, N. Ikemoto, J. Ishikawa, S. Nishimoto, *J. Biomed. and Mater. Res.*, 44 (1999) 22.

[3] H. Tsuji, H. Sato, T. Baba, S. Ikemura, Y. Gotoh, J. Ishikawa, *Nucl. Instr. and Meth.* B 166/167 (2000) 815.

[4] H. Tsuji, H. Sasaki, H. Sato, Y. Gotoh, J. Ishikawa, *Nucl. Instr. and Meth.* B 191 (2002) 815.

[5] R. Ikeguchi, R. Kakinoki, T, Matsumoto, H. Tsuji, J. Ishikawa, T. Nakamura, *Development Brain Research,* 140(2003)127.

[6] H. Tsuji, M. Izukawa, R. Ikeguchi, R. Kakinoki, H. Sato, Y. Gotoh, J. Ishikawa, *Nucl. Instr. and Meth,* B 206(2003)507.

[7] H. Tsuji, J. Ishikawa, S. Ikeda, Y. Gotoh, *Nucl. Instr. and Meth.* B 127/128 (1997) 278.

[8] H. Tsuji, Y. Gotoh, J. Ishikawa, *Nucl. Instr. and Meth.* B 141 (1998) 645.

[9] H. Tsuji, T. Taya, J. Ishikawa, T. Takagi, "Proceedings of the International Ion Engineering Congress, ISIAT'83 & IPAT'83", Vol.1, Ed. by T. Takagi, Institute of Electrical Engineers of Japan, Tokyo (1983) pp.141-146.

[10] G. Lundborg, L.B. Dahlin, N. Danielsen, H. Gelberman, F.M. Longo, H.C. Powell, *Exp. Neurol.* 76 (1982) 361.

[11] R.D. Madison, C.F. Da Silva, P. Dikkes, T.H. Chiu, R.L. Sidman, *Exp. Neurl.* 76 (1985) 767.

[12] L.R. Williams, N. Danielsen, H. Muller, S. Varon. *J. Comput. Neurol.* 264 (1987) 284.

[13] R.D. Madison, C.F. Da Silva, P. Dikkes, *Brain Res.* 447 (1988) 325.

[14] I.V. Yannas, D.P. Orgill, J. Silver, T.V. Norregard, N.T. Zervas, W.C. Schoene, Transaction of the Sixth European Conference of Biomaterials, Paris, (1989) pp. 4 -6.

[15] R. Kakinoki, N. Nishijima, Y. Ueba, M. Oka, T. Nakamura, *Neurosci. Res.* 23 (1995) 35.

[16] R. Kakinoki, N. Nishijima, Y. Ueba, M. Oka, T. Yamamuro, T. Nakamura, *J. Neurosurg. Sci.* 42 (1998) 11.

(Received October 9, 2003; Accepted January 20, 2004)