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Protein Adsorption Properties on Silicone Rubber Modified by Carbon Negative-Ion Implantation

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The adsorption properties of proteins such as laminin (LN), fibronectin (FN) and gelatin on the carbon negative-ion implanted silicone rubber sheet (SR) including their effects of these protein-coated modified SR on patterning nerve-cell culture were investigated. The implantation conditions were fixed at 3×10^{15} ions/cm^2 and 10 keV. The concentrations of LN in PBS, FN in PBS and gelatin in de-ionized water (DIW) were 0.5-50, 0.5-10 and 5-10^3 μg/ml, respectively. The adsorption properties of proteins on SR sheet implanted as a half moon shape were evaluated by XPS. After 4-day *in vitro* culture of the nerve-like cell of rat adrenal pheochromocytoma (PC12h) on the protein-coated surfaces of SR implanted through the micro-pattern mask with slits of 50-μm width, the phase contrast micrographs show that the suitable protein for patterning the attachment of nerve cell was FN at 1 μg/ml of concentration, corresponding to high ratio of nitrogen adsorption between implanted and unimplanted regions that was 1.9. As coated with 1μg/ml of LN, cells attached over all areas. While coating with 1 mg/ml of gelatin, no cell attached.
Key words: Negative ion implantation, Protein adsorption, Cell attachment, Silicone rubber, PC12h

1. INTRODUCTION

Biocompatible improvements of silicone rubber (SR) surface for nerve-cell affinity were investigated by carbon negative-ion implantation [1-5] since this technique has a good advantage of charge-up free [6-8]. The improved-attachment properties on the polymeric surfaces modified by carbon negative-ion implantation were from the present of hydrophilic bonds such as C-O(H) and C=O after implantation [1-5]. Then, the cell attachment properties could be controlled by the surface property through controlling of the implantation condition [6]. Tsuji *et al.* presented the suitable implantation condition for the nerve cell-attachment properties on the implanted region of SR modified by carbon negative-ion implantation [1-5]. However, the patterned nerve cell-attachment on SR still was not good since lack of cell attachment on some parts of the implanted surface. This indicates the weak attachment force of cells on some areas of the implanted region. The attachments of cells on the modified surfaces relate to the suitable protein adsorption on the hydrophilic surfaces. In cell culture, proteins such as laminin, collagen including gelatin, fibronectin, vitronectin, etc. of an extracellular matrix (ECM) lies between the cell body and the surface. Cells required such ECM as a 'footing' site for attachment on the surface. Generally, cells can produce such ECM by themselves and some of these ECM also are found in the culture medium with serum. However, the present of ECM sometimes is not enough for the cell requirement. Therefore, the pre-coated modified surface by the suitable protein should improve the uniform of patterned attachment of nerve cells.

In this present, the adsorption properties of proteins, such as laminin, fibronectin and gelatin, and the suitable protein-coated surfaces for more improvement of the nerve cell-attachment properties on the C-implanted SR were investigated.

2. EXPERIMENT

Surfaces of silicone rubber sheet (SR, Wacom Electric. com Inc., Japan) were modified by carbon negative-ion implantation. Carbon negative ions produced in a cesium sputter-type heavy negative-ion source (NIABNIS) [9-10] were mass-separated and transported to an implantation chamber. The carbon negative-ion beam of 11.28 mm in diameter was implanted to SR at an applied energy of 10 keV and dose of 3×10^{15} ions/cm^2. A current density was kept less than 400 nA/cm^2 under residual gas pressure less than 6×10^{-4} Pa. For the ECM adsorption, the adsorption properties of proteins, which relate to the cell affinity, such as laminin (LN, L2020, Sigmal-aldrich Inc.), fibronectin (FN, F1141, Sigmal- aldrich Inc.) and gelatin (GEL, G1890, Sigmal-aldrich Inc) on the C-implanted SR were investigated by XPS. The concentrations of LN in phosphate buffered saline (PBS), FN in PBS and gelatin in de-ionized water (DIW) were 0.5-50, 0.5-10 and 5-10^3 μg/ml, respectively. The as-implanted SR sheets with a half moon shape of implanted region were dipped in the protein solution with these concentrations for 2h at 37°C, and they were then rinsed by DIW for 2-3 times. After that samples were completely dried in the oven at 37°C for 30 minutes before evaluation by XPS (AXIS-165s, Shimadzu). XPS narrow spectra of N on the implanted and unimplanted regions were measured with an X-ray

source of monochromatic AlKα (1486.6 eV) at a relatively weak intensity so as to avoid modifying the surface state by irradiation.

For observation of the nerve-cell attachment properties on the protein-coated modified SR, the samples were implanted through a micro-pattern mask of many slit apertures 50-μm width and 70-μm spacing, and each C-implanted sample was then fixed into a 35-mm dish (Non-treated polystyrene dish, Corning) by using silicone glue. After 2 days, all dried dishes were sterilized by 70% ethanol, rinsed three times with the sterilized DIW and rinsed once with PBS before protein coating and before cell culture. The samples were coated by the solutions of laminin, fibronectin and gelatin with concentrations of 1, 1 and 10^3 μg/ml, respectively. After keep the protein coated samples in the incubator for 2h, the samples were once rinsed by DIW and PBS. Nerve-like cells of rat adrenal pheochromocytoma (PC12h) about 2.5×10^5 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 4 days under 5% CO_2 at 37°C in incubator, as well as on the uncoated C-implanted SR as a control. Then, their cell-attachment properties on the protein coated surfaces were observed by phase contrast microscope (CK2, Olympus).

3. RESULTS AND DISCUSSION

3.1 Protein adsorption by XPS

The XPS survey spectra in Fig. 1 show the increases of intensities for carbon and oxygen after ion implantation. The carbon intensity was increased due to the doping atoms from the ion implantation. The oxygen intensity was increased due to the formation of the oxygen functional groups of C-O and C=O on the ion-induce defect after ion bombardment. The formation referred to the hydrophilic property of the implanted region as described in the introduction. Generally, the necessary proteins for cell attachment are absorbed on the hydrophilic surface [11]. Therefore, the ECM adsorption on the implanted region should be higher than that of the unimplanted region.

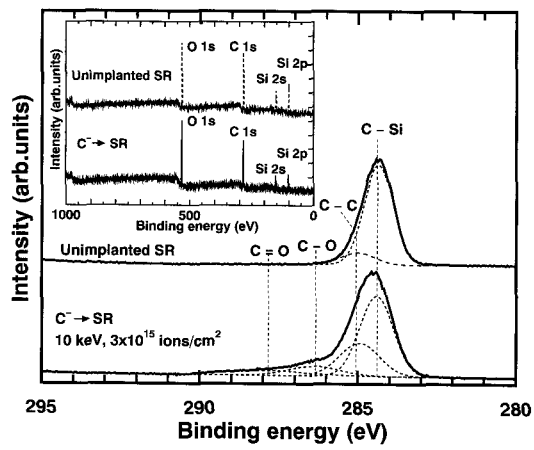


Fig. 1. C 1s narrow spectra of the unimplanted and implanted regions of the C-implanted SR (C-SR) at 10 keV and 3×10^{15} ions/cm^2, including survey spectra.

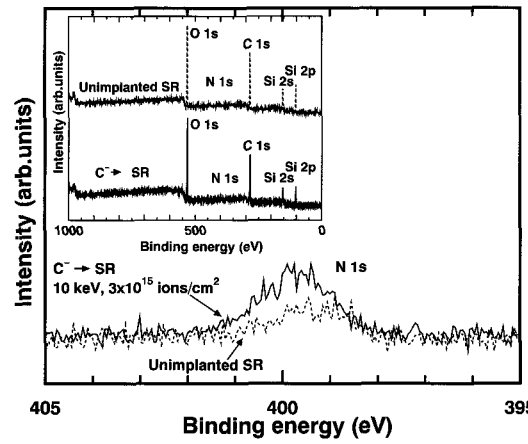


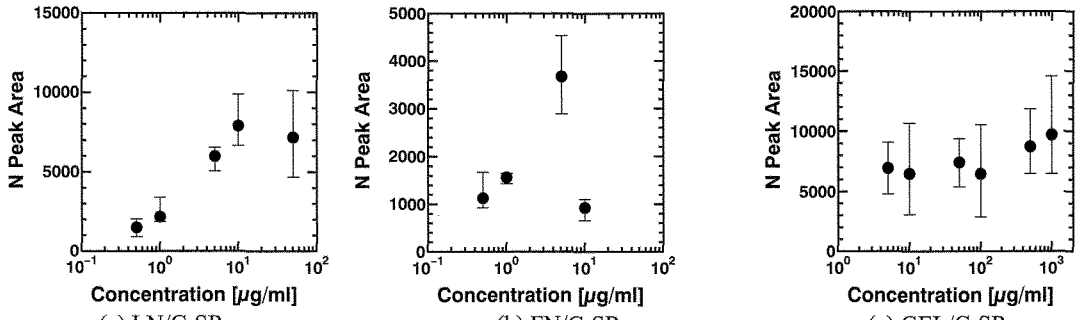
Fig. 2. N 1s narrow spectra of the unimplanted and implanted regions of the C-implanted SR (C-SR) at 10 keV and 3×10^{15} ions/cm^2 after coating with laminin at concentration of 1μg/ml, including survey spectra.

Fig. 2 shows the XPS spectra for the C-implanted SR after coating with laminin at concentration of 1 μg/ml. After coating, the peak of N 1s narrow appeared in XPS survey spectra from the protein adsorption. The adsorption of nitrogen atoms of the implanted region was larger than that of the unimplanted region. The amounts of protein adsorption on the C-implanted SR were evaluated by the calculation of the N peak area of the XPS narrow spectra of nitrogen since there is no nitrogen atom in the monomer structure of SR for both cases of before and after implantation.

Based on XPS analysis, the amounts of nitrogen adsorption from the amino acid in each protein type on the implanted regions of C-implanted SR as a function of protein concentration are shown in Fig. 3.

The amounts of nitrogen adsorption on the implanted regions of C-implanted SR at 10 keV and 3×10^{15} ions/cm^2 increased as a function of the concentrations of laminin, fibronectin and gelatin as shown in Figs 3(a), 3(b) and 3(c), respectively. From Fig. 3(a), the amount of nitrogen adsorption for laminin increased as increase in the concentration in the range of 0.5 - 10 μg/ml from 1490 to 7890 before decreased to 7170 at the concentration of 50 μg/ml. The amount of fibronectin also increased as increase in the concentration in the range of 0.5 - 5 μg/ml from 1130 to 3670 before rapidly decreased to 930 at the concentration of 10 μg/ml. Another amount of gelatin also increased as increase in the concentration from 6470 to 9700. The maximum adsorption of laminin, fibronectin and gelatin were at 10, 5 and 1000 μg/ml, respectively. The amount of nitrogen adsorption from the nitrogen atom in the amino acid of gelatin was largest, but their errors of all concentrations also were largest. The nitrogen adsorptions of laminin on C-implanted SR were also good, excepting at high concentration that the large error occurred. The nitrogen adsorptions of fibronectin on C-implanted SR were not good.

The large error in case of gelatin adsorption may be from the adhesive force between the amino acids (N-H) of gelatin and the hydrophilic bonds (C-O(H) and C=O) of C-implanted SR. Gelatin is obtained from the deterioration of collagen, which has many hydrophilic-types of amino acids. So, the amino acids of gelatin look-like that of



(a) LN/C-SR (b) FN/C-SR (c) GEL/C-SR

Fig. 3. N peak area on the implanted regions of C-implanted SR (C-SR) as a function of the concentration of: (a) laminin (LN); (b) fibronectin (FN); and (c) gelatin (GEL).

collagen, but the adhesive force was different. The adhesive force between amino acid bonds and surface comes from the hydrogen bond that is easily destroyed by water force [12]. That means too large amount of nitrogen adsorption in someplace makes the detachment easily to happen. Therefore, the errors of gelatin at the same concentration were so large. The problem of large errors for the amount of nitrogen adsorption also happened in the laminin at high concentration such as 10 and 50 μg/ml. Laminin is a basement membrane for cell attachment that has many domains to bind to other proteins. That means it is composed of many hydrophilic- and hydrophobic-type of amino acids to increase the adhesive force in binding together and to other protein. However, the hydrogen bonding between the large amount of amino acids and the hydrophilic bonds of C-implanted SR can be also broken by the force of water. Then, the amount of nitrogen adsorption from laminin decreased at high concentration of laminin. These reasons of decrease in the amount of nitrogen adsorption from the amino acids of laminin and gelatin could also be used to describe that why the amount of nitrogen adsorption for fibronectin decreased.

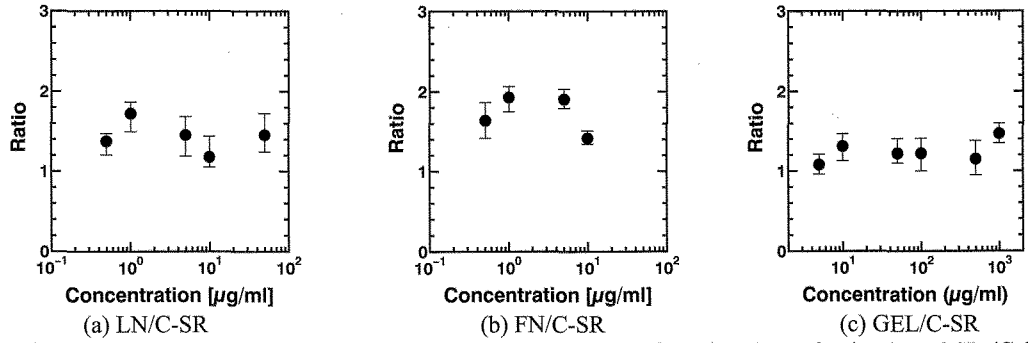
Based on calculation of N peak areas, the ratios of the amount of nitrogen adsorption from each protein between the implanted and unimplanted regions of C-implanted SR at 10 keV and 3×10^{15} ions/cm^2 as a function of concentration are shown in Fig. 4. The adsorption ratios for these three proteins were different. The adsorption ratios of laminin increased as increase in the concentrations of 0.5-1 μg/ml from 1.38 to 1.72 and decreased in the concentration range of 1-10 μg/ml to 1.18 before increased again to 1.46 at concentration of 50 μg/ml as shown in Fig. 4(a). The maximum ratio for laminin adsorption was at 1 μg/ml. The adsorption ratios of fibronectin increased as increase in the concentrations of 0.5-1 μg/ml from 1.64 to 1.93 and saturated in the concentration range of 1-5 μg/ml at 1.91 as shown in Fig. 4(b). After this concentration, the ratio decreased to 1.42. The maximum ratio for fibronectin was also at 1 μg/ml. Fig. 4(c) shows the adsorption ratios of gelatin that increased as increase in the concentrations of 5-1000 μg/ml from 1.1 to 1.47. The maximum ratio for gelatin adsorption was at 1000 μg/ml.

By trade-off between the amount of nitrogen adsorption and their adsorption ratio at each concentration for each protein, the considerably suitable concentrations of laminin, fibronectin and gelatin to pre-coat the C-implanted SR at 10 keV and 3×10^{15} ions/cm^2 for improving the selective cell-attachment pattern should be at 1, 1 and 1000 μg/ml, respectively.

3.2 Nerve-cell attachment

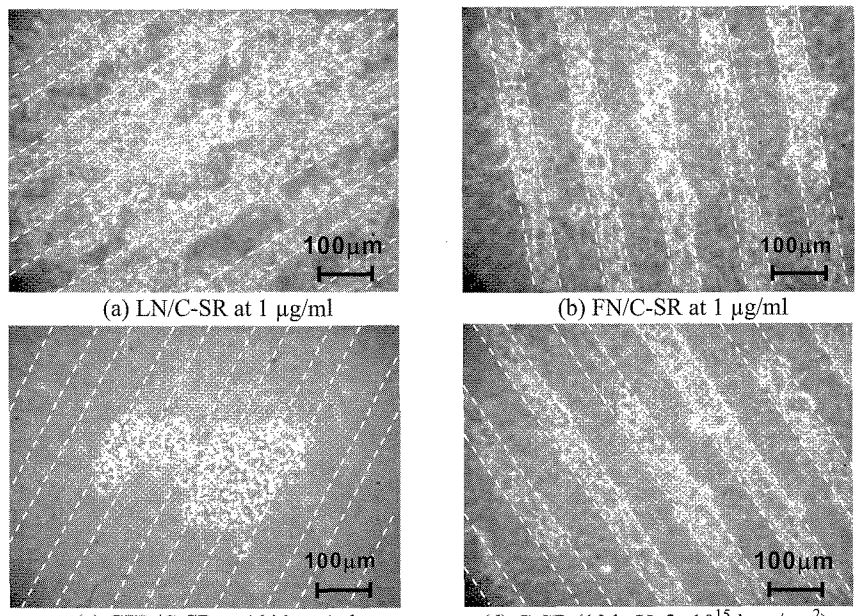
Fig. 5 shows that the cell-attachment properties on all protein-coated surfaces of C-implanted SR at 10 keV and 3×10^{15} ions/cm^2 depended on the protein type. A lot of cells with non selective attachment pattern were found on the laminin-coated surface as shown in Fig. 5(a), where the brightness areas correspond to the cell attachment areas. Almost cells were found on the implanted region, where corresponds to the narrow region between dashed-lines. The reason may be from the adsorption ratio value of the nitrogen adsorption amounts between the implanted and unimplanted regions, from the excess of nitrogen adsorption amount on the unimplanted region and from the specific strong-adhesive force properties to surface of laminin.

Comparing to the cell-attachment properties on laminin-coated surfaces, a lot of cells selectively attached as a definite pattern on the fibronectin-coated surface of



(a) LN/C-SR (b) FN/C-SR (c) GEL/C-SR

Fig. 4. Adsorption ratio between N peak area on the implanted and unimplanted regions of C-implanted SR (C-SR) as a function of the concentration of: (a) laminin (LN); (b) fibronectin (FN); and (c) gelatin (GEL).



(a) LN/C-SR at 1 μg/ml (b) FN/C-SR at 1 μg/ml

(c) GEL/C-SR at 1000 μg/ml (d) C-SR (10 keV, 3×10^{15} ions/cm^2)

Fig. 5. Phase contrast micrograph of PC12h cells after 4 days culture on SR implanted at 10 keV and 3×10^{15} ions/cm^2 coated with proteins of: (a) laminin at 1 μg/ml; (b) fibronectin at 1 μg/ml and (c) gelatin at 1000 μg/ml, as well as (d) on control.

C-implanted SR as shown in Fig. 5(b). The reason may be from the high adsorption ratio (1.93) and from the suitable amount of nitrogen adsorption on the implanted region (2170) without the excess of this amount on the unimplanted region. While very small amount of attached cells were on the gelatin-coated surface of C-implanted SR as shown in Fig. 5(c). Small cell attachments were found on both of the implanted and unimplanted region. The reason may be from the low adsorption ratio and from too excess of nitrogen adsorption amount on both implanted and unimplanted region that make the detachment of protein out from the surface happen. Thus, cells could not attach on this surface.

From all investigation, the suitable protein and concentration for improvement of selective nerve-cell-attachment pattern on the C-implanted SR at 10 keV and 3×10^{15} ions/cm^2 was fibronectin at 1 μg/ml.

4. CONCLUSION

Adsorption properties of proteins such as laminin (LN), fibronectin (FN) and gelatin on the carbon negative-ion implanted silicone rubber sheet (SR) including their effects of these protein-coated modified SR on pattering nerve-cell culture were investigated. Based on XPS analysis, the suitable protein concentration to obtain the good adsorption properties of both the amount of nitrogen adsorption and the high adsorption ratio for laminin, fibronectin and gelatin were 1, 1, 1000 μg/ml, respectively. After 4-day culture of the PC12h cells, the surfaces coated by laminin and gelatin were not suitable for the improvement of nerve-cell-attachment pattern. The suitable protein for improvement of the selective nerve-cell-attachment pattern on the C-implanted SR at 10 keV and 3×10^{15} ions/cm^2 was fibronectin at 1 μg/ml, corresponding to the high adsorption ratio at 1.93 and the small enough amount of nitrogen adsorption with 2170 on the implanted region.

5. REFERENCES

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