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Mesenchymal Stem Cell Attachment Properties on Silicone Rubber Modified by Carbon Negative-Ion Implantation

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Biocompatibility of silicone rubber sheet (SR) was improved by the carbon negative-ion implantation for the attachment of mesenchymal stem cells (MSC). The carbon negative ions were implanted at 10 keV and various ion doses from 0.01 – 10 ($\times 10^{15}$) ions/cm^2 through the micro-pattern mask with aperture slits of 50-μm width for study of the selective attachment property. Contact angles of the modified SR at the same conditions without the micro-pattern mask were measured by water drop and air bubble. After 25-day *in vitro* culture of MSC from rat bone marrow on the implanted SR, the phase contrast micrographs showed the attachment of cells only on the stripe regions implanted with doses from 1 – 10 ($\times 10^{15}$) ions/cm^2. The good conditions for cell attachment are 3 and 10 ($\times 10^{15}$) ions/cm^2 of ion doses corresponding to the low contact angle, which is 69° and 61°. The fluorescence micrographs also confirmed that the position of cell nucleus and the actin filament on the implanted region at these implantation conditions. As implanted at low dose, no cell attached and cells very weakly attached.
Key words: Negative ion implantation, Cell attachment, Silicone rubber, Contact angle, Mesenchymal stem cells

1. INTRODUCTION

The negative ion implantation such as silver and carbon elements were used to modify the surface of polymeric material [1-8] because the good advantage of "charge up free" [9-11]. The carbon element is mainly component of polymer materials and more familiar to cells. It matches to the lattice of the polymer and also is harmless for the cell affinity and the living body. Therefore, the implantation with the carbon negative ions was selected to improve the biocompatibility of the polymeric surfaces by modification of the physical surface properties [2-8]. As previous work, we obtained the formation of oxygen functional groups such as carbonyl and hydroxyl on the ion-induced defects on the surface after ion implantation [3, 7]. The negative polar characteristics in these oxygen functional groups refer to the hydrophilic properties due to their polarized dipole. The Lowering of contact angle after implantation presented the more hydrophilic property, resulting in the improved-attachment properties of nerve-like cells such as PC12h cells [6-8]. In fact of cell culture, however, not only surface property that affects on the attachment property, but also other factors such as the cell lines and the extracellular matrix proteins (ECM) do [3,4]. In case of the development of an artificial neuron network and the improvement of the nerve regeneration performance, the culture of real nerve-cell or neuron-differentiated cells on the C-implanted surface is necessary. One of interesting cell lines to culture on the C-implanted silicone rubber to study this purpose is the mesenchymal stem cell (MSC) derived from bone marrow. This cell is worldwide used in the clinical researches since it can differentiate into many cell lines such as bone, muscle, cartilage, marrow stromal, neuron and etc [12].

In this present, the contact angle of water at various circumstances and the attachment properties of mesenchymal stem cell on the silicone rubber sheet modified by the carbon negative-ion implantation were investigated.

2. EXPERIMENT

Silicone rubber sheets (SR, Wacom Electric co., ltd, Japan) with 0.5 mm in thickness were implanted by carbon negative ions for surface modification. Carbon negative ions produced in a cesium sputter-type heavy negative-ion source (NIABNIS) [13-14] were mass-separated and transported to an implantation chamber. The carbon negative-ion beam of 11.28 mm in diameter was implanted to the sheets at various ion doses of 0.01, 0.03, 0.1, 0.3, 1, 3, and 10 ($\times 10^{15}$) ions/cm^2 for applied ion energy of 10 keV. The current density was kept less than 400 nA/cm^2 under the residual gas pressures less than 6×10^{-4} Pa. Physical surface properties of modified surface, which relate to the cell affinity, such as a wettability were simply evaluated by contact angle measurement. The contact angle of pure water de-ionized by a filter was measured by water drop method as implanted and by air bubble method after dipping in pure de-ionized water (DIW) for 0, 2, 24 and 48 h. The air bubble method was done by putting a small bubble less than 1-mm diameter on the sample during dipping in DIW. For observation of MSC-attachment on the modified SR, the samples were implanted through a micro-pattern mask of many slit apertures 50-μm width and 70-μm spacing with 5×10 mm^2 in area. All C-implanted SR sheets were sterilized by 70% ethanol, rinsed three times with the sterilized DIW and set into a 35-mm dish (Non-treated

polystyrene dish, Corning). Before cell culture, all of them were rinsed once with the phosphate buffered saline (PBS). The passage number 3 of MSCs, which were derived from rat bone marrow, was chosen to this study. Cells were lifted by incubation with trypsin (0.25%) and 1mM EDTA at 37°C for 4-5 min. Cells were then cultured on the C-implanted SR surfaces in Dulbecco's modified Eagle's medium (DMEM, Nissui, Japan) containing 5% heat-inactivated horse serum (HS, Gibco, New Zealand), 5% fetal bovine serum (FBS, Trace Scientific Ltd., Australia), sodium hydrogen carbonate (1.8 mg/ml, Wako, Japan) with antibiotic of penicillin G and streptomycin for 20-25 days under 5% CO_2 at 37°C in incubator. The culture of cell on the tissue cultured polystyrene dish (TCPS, Corning, USA) was used as a control. The culture medium of each dish was exchanged by the fresh medium pre-warmed to 37°C every 4 days. Attachment properties of MSC on C-implanted SR were observed by phase-contrast microscope (CK2, Olympus). Finally, the cells were stained with 4', 6-Diamidino-2-phenylindole (DAPI, Sigma-aldrich, Japan) and Texas Red-X phalloidin (TrX-phalloidin, Molecular probes, Japan) to investigate their nucleus and their actin filament (f-actin) by fluorescence microscope (BX50, Olympus).

3. RESULTS AND DISCUSSION

3.1 Contact angle

The contact angles of DIW on the C-implanted SR sheet at 10 keV as the function of ion dose measured by the water drop method as implanted and by the air bubble method after dipping in DIW for 0, 2, 24 h are shown in Fig. 1.

The contact angles from water drop method gradually decreased as increase in the ion dose and saturated in the dose range of 1–3 ($\times 10^{15}$) ions/cm^2 as shown in Fig. 1(a). Comparing to the unimplanted SR, the angle values decreased from 100° to the lowest angle of 86° at 3×10^{15} ions/cm^2. After this dose, the angle increased with a few degrees from the lowest angle. While the angles from air bubble method at 0h dipping in DIW increased at very low implantation doses of 1–3 ($\times 10^{13}$) ions/cm^2 from 90° to 96°, and the angles after this very low dose gradually decreased to the lowest angle of 61° as increase in the ion dose as shown in Fig. 1(b). The high angle value at very low implantation dose showed the hydrophobic property of surface from cleaning by sputtering. The more lowering of angle at high implantation dose of 1×10^{16} ions/cm^2 might be from bonding between the molecules of water and bulk material of SR.

The time dependence of the contact angles on the C-implanted SR as the function of ion dose at 10 keV after dipping in the DIW for 0, 2, and 24h are also shown in Fig. 1(b). After dipping all implanted SR sheets in DIW the angle rapidly decreased for 3°-8° within first 2h, excepting at very low doses of 1–3 ($\times 10^{13}$) ions/cm^2 that decreased for 2°–4°. Then, angle gradually decreased for 9°–17° until saturated during dipping time from 2 to 24h. The lowest angle was 47° at 1×10^{16} ions/cm^2 after 24h dipping in DIW.

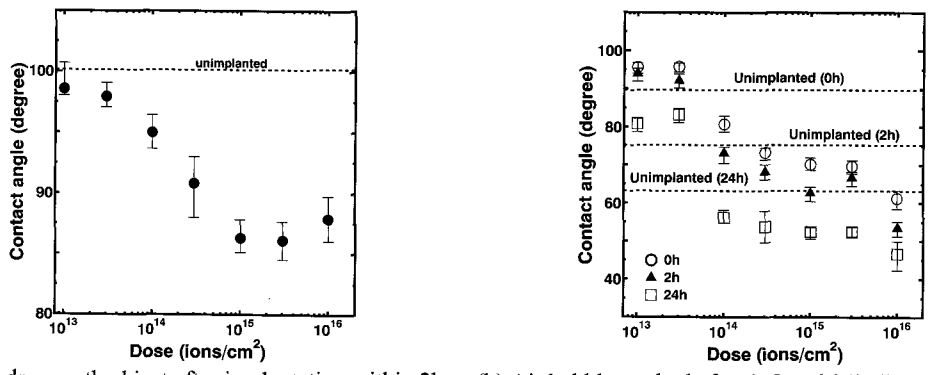
Therefore, the considerably suitable dose condition to obtain high possibly MSC-attachment properties should be in the range of 1–10 ($\times 10^{15}$) ions/cm^2.

3.2 Mesenchymal stem cell attachment

The shape and size of MSC on control were unclear because of the spread of the cell-body and some differentiation on the surface after attachment as shown in the upper right side of Fig. 2(a). Moreover, the transparence of this cell leads to the inconvenient observation with the small number of cells. After culture of MSC for 3 days, the growth in number of MSC and their attachment were enough to facilitate the observation of the attachment property on the patterned-bulk material such as silicone rubber.

Based on phase-contrast optical micrograph, typical phase contrast micrograph of MSC cultured for 3 days on the C-implanted SR at 1×10^{13} and 1×10^{14} ions/cm^{2}as shown in Fig. 2, where the dashed ovals correspond to the areas of attached cells.

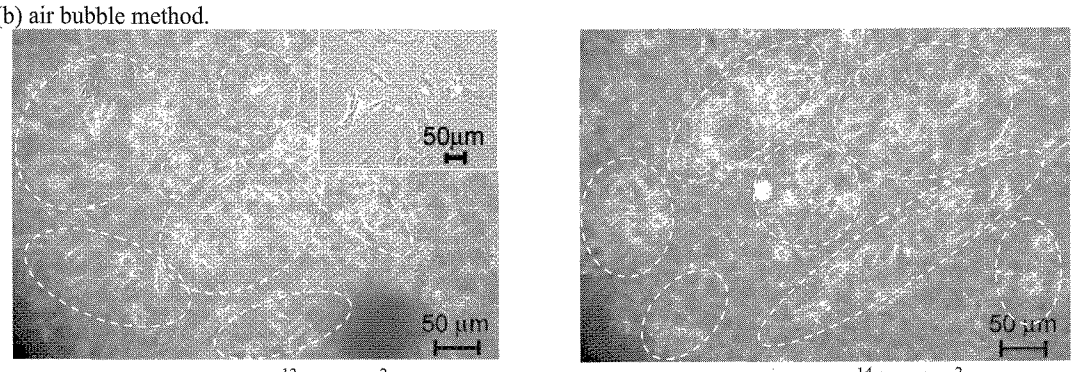
Comparing to the normal attachment of MSC on control, no spread-attachment and differentiation was found on the C-implanted SR at low doses of 1×10^{13} and 1×10^{14} ions/cm^2. At these low implantation doses less than dose order of 10^{15} ions/cm^2, the stripe of implanted region cannot be seen. However, we can confirm that some cells attached only on the implanted region or the edge of implanted region due to the lowering of contact angle value on the implanted region. Excepting, at dose order of 10^{13} ions/cm^2 (Fig. 2(a)), small amount of attached cells might be on the edge of implanted region



(a) Water drop method just after implantation within 2h (b) Air bubble method after 0, 2 and 24h dipping in DIW

Fig. 1. Contact angle of C-implanted SR at 10 keV as a function of ion implantation dose measured by (a) water drop

and (b) air bubble method.



(a) C/SR at 1×10^{13} ions/cm^2 (b) C/SR at 1×10^{14} ions/cm^2

Fig. 2. Phase contrast micrographs of MSC after 3-day culture on the C-implanted SR (C/SR) at 10 keV with: (a) 1×10^{13}; and (b) 1×10^{14} ions/cm^2.

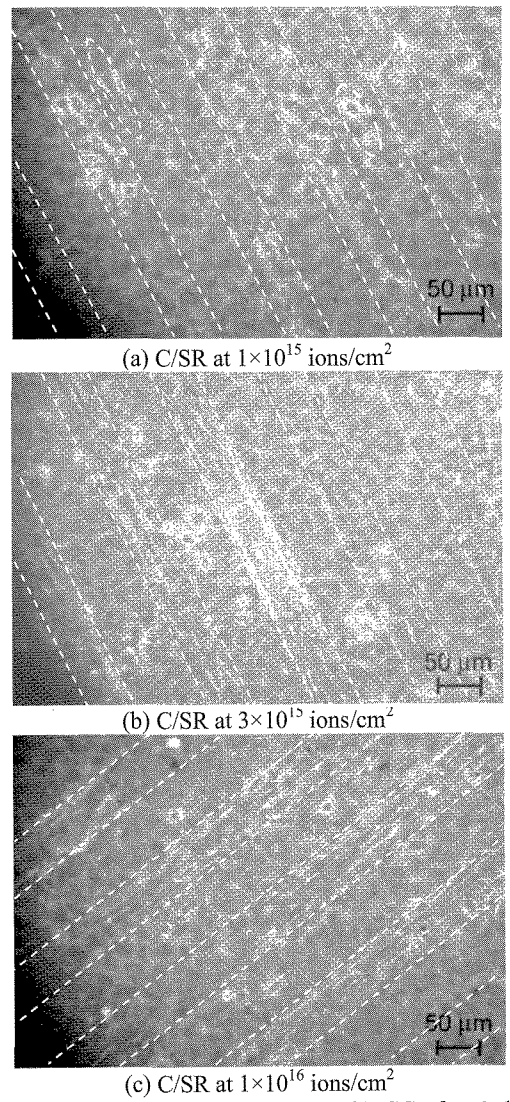
since the observable cells were on the surface, which is the ion beam implanted area. Similar to these results, small amount of cells randomly attached on the SR surfaces implanted at 10 keV with low doses in the range of 0.1 – 3 ($\times10^{14}$) ions/cm^2. The attachments of MSC at these low implantation doses were not good, and the cells detached from the implanted surface when only the movements of culture medium were happened. These weak-attachments with very small amount of MSC on the C-implanted SR also correspond to the hydrophobic surface at dose order of 10^{13} ions/cm^2 and to the little lowering of contact angle as a little to 68° at dose order of 10^{14} ions/cm^2 after dipping in DIW for 2 h.

The selective attachments of MSC were found on the C-implanted SR at doses of 1 – 10 ($\times10^{15}$) ions/cm^2 as shown in Fig. 3.

The attachments on the implanted regions, where the implanted regions are presented by the narrow regions of 50 μm in width between dashed-lines, of these implantation doses correspond to the lowering of contact angle to 54° – 67° after 2h dipping in DIW. At dose of 1×10^{15} ions/cm^2, some cells started to selective attach on the implanted region as shown in Fig. 3(a). However, the MSC attachment property of C-implanted SR at this dose was worse than those of the C-implanted SR at 3–10 ($\times10^{15}$) ions/cm^2 as shown in Figs. 3(b) and 3(c), respectively. Only 60% of seeded cells attached on the implanted regions of the C-implanted SR at 1×10^{15} ions/cm^2, and their adhesive force was quite weak, because about 40% of these attached cells were detached after replacement of the fresh culture medium. In the other hand, the cells on C-implanted SR at 3–10 ($\times10^{15}$) ions/cm^2 still attached after the fresh medium replacement. Moreover, at these implantation doses, the spread of cells were along the implanted region and some look-liked differentiation of cells were found. Therefore, the suitable doses for selective MSC attachment with strong adhesive force were 3–10 ($\times10^{15}$) ions/cm^2.

The observations of the attachment and differentiation of cells only by the phase-contrast optical micrographs are inconvenient since the transparence of MSC and many patterns in SR bulk. The exactly attached-position of each cell and the definite shape were not explained. So, the fluorescent microscopy for observation of the microorganism such as nucleus and f-actin in cell body can help to explain the finite position and shape. Fig. 4 shows the fluorescent micrographs of MSC on the C-implanted SR at 10 keV and 3×10^{15} ions/cm^2 after 25-day culture.

Although, the cell attachments on the C-implanted SR surface were difficult to observe by the optical micrograph as shown in Fig. 4(a), their alignments of



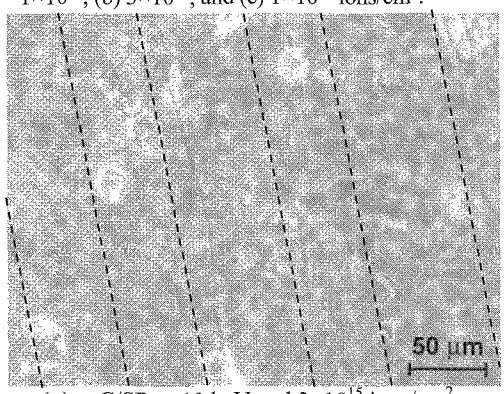
(a) C/SR at 1×10^{15} ions/cm^2

(b) C/SR at 3×10^{15} ions/cm^2

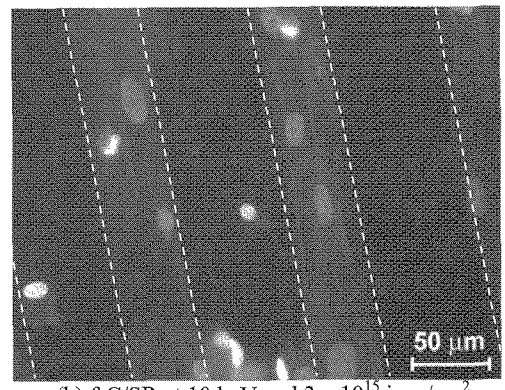
(c) C/SR at 1×10^{16} ions/cm^2

Fig. 3. Phase contrast micrographs of MSC after 3-day culture on the C-implanted SR at 10 keV with: (a)

1×10^{15}; (b) 3×10^{15}; and (c) 1×10^{16} ions/cm^2.



(a) p-C/SR at 10 keV and 3×10^{15} ions/cm^2



(b) f-C/SR at 10 keV and 3 x 10^{15} ions/cm^2

Fig. 4. Fluorescent micrographs of MSC stained on the C-implanted SR at 10 keV with 3×10^{15} ions/cm^2 after 25-day culture: (a) optical micrograph of cells (p-C/SR); and (b) fluorescent micrograph of the nucleus and alignment of f-actin (f-C/SR).

nucleus and f-actin at the same point could clearly show that cells almost attached and spread along the implanted region as shown in Fig. 4(b), where the bright spots and the bright stripes with lines correspond to the nucleus and the f-actin, respectively. Some neurite-like f-actins were also found. As similar to this result, the nucleus and the f-actin of MSC on the C-implanted SR at $1x10^{16}$ ions/cm^2 also attached and spread along the implanted regions (data not shown).

4. CONCLUSION

The mesenchymal stem cell attachment properties on the surface of silicone rubber modified by carbon negative-ion implantation at 10 keV were observed. After implantation, the surface wettability of silicone rubber became to the hydrophilicity. The air bubble method showed the lowering of contact angles after dipping in DIW for 0 and 24h from 61° to about 47° at implantation dose of 1×10^{16} ions/cm^2, excepting at dose of 1–3 ($\times10^{13}$) ions/cm^2 that became to the hydrophobicity. After 3-day culture, the suitable implantation doses at 10 keV for the selective MSC-attachment property with strongly adhesion force were at 3 – 10 ($\times10^{15}$) ions/cm^2, which correspond to the low contact angle less than 53° after dipping in DIW for 24 hours. The observation of the nucleus and f-actin by fluorescent microcopy after 25-day culture also confirmed the selective attachment and differentiation of MSC on the implanted regions of these both implantation doses and showed the some differentiation to the neuron-like cells.

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