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Adhesion of human osteoblast-like cells (Saos-2) to carbon nanotube sheets

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Abstract. Carbon nanotubes (CNTs) exhibit excellent cell proliferation properties, which can serve as a scaffold for cell culturing. However, there are only a few reports on adhesion of osteoblast-like cells to a CNT sheet. In this study, we investigated adhesion of osteoblast-like cells to single-walled carbon nanotube (SWNT) and multi-walled carbon nanotube (MWNT) sheets and compared these adhesions with that on a cell culture polystyrene dish by using a cell adhesion test and a scanning electron microscope. The MWNT sheets exhibited faster adhesion of cells at an initial stage than SWNT sheets and cell culture polystyrene dish. The number of attached cells on the MWNT sheets seemed to be greater than on SWNT sheets and cell culture polystyrene. Moreover, the MWNT sheets exhibited both high speed and good capacity for cell adhesion. However, the surface of the MWNT sheets was such that it facilitated cell adherence but hindered the spreading of the attached cells. Interestingly, cell adhesion to CNT sheets was significantly influenced by pre-coating with serum. These results indicate that CNT sheets would play an important role in adsorption of serum proteins, which would consequently facilitate cell adhesion, and that the MWNT sheets have a high cell adhesiveness.

Keywords: Carbon nanotubes, osteoblast-like cells, cell adhesion, serum, spreading

1. Introduction

Carbon nanotubes (CNTs) have been attracting considerable attention because of their unique physical properties and potential for a variety of applications [1]. Our recent studies involving *in vitro* experiments demonstrated excellent properties of CNTs to serve as a scaffold for cell culturing, such as cell adhesion, proliferation, and protein adsorption [2,3]. On contact of CNT scaffolds begin with cells, cell adhesion occurs, followed by subsequent spreading, migration, proliferation, and differentiation of osteoblasts and bone formation. There are only a few reports on adhesion of osteoblastic cells to CNT scaffolds; in particular, the effect of serum on cell adhesion has not been studied. Casey *et al.* found that components of both the cell culture medium and the fetal bovine serum (FBS) growth supplement interact with CNTs, and their interactions affect cell responses [4]. Aoki *et al.* reported that serum protein adsorption by CNT scaffolds might enhance cell proliferation [2,3]. One possibility is that cell adhesion to CNT scaffolds might be greatly influenced by conditions of serum protein adsorption in a medium.

In this study, we investigated the adhesion of human osteoblast-like cells (Saos-2) to single-walled carbon nanotube (SWNT) and multi-walled carbon nanotube (MWNT) sheets, and compared these

adhesion with that on a cell culture polystyrene dish, which is a widely used substrate. Here, we report that (i) MWNT sheets exhibit faster adhesion of cells at an initial stage than SWNT sheets and cell culture polystyrene dish, (ii) MWNT sheets exhibit both high speed and good capacity for cell adhesion, and (iii) adhesion of osteoblast-like cells to CNT sheets is drastically influenced by pre-coating with serum.

2. Materials and methods

2.1 Preparation of CNT Sheets

Purified SWNTs (0.8–2.5 nm in diameter) were purchased from Meijo Nano Carbon Co. Ltd. (Nagoya, Japan) and synthesized using the arc discharge method [5]. MWNTs were purchased from NanoLab Inc. (Brighton, MA) and synthesized using the chemical vapor deposition technique. The MWNTs (average diameter, 30 nm) were purified using a previous method [6]. The SWNTs had >95wt% purity and contained <5wt% amorphous carbon as the dominant impurity, while the MWNTs had >98wt% purity and contained <2wt% amorphous carbon. CNT sheets were prepared using our

previous methods [2,3]. In order to prepare CNT sheets, 100 mL of CNT solution in 99.5% ethanol (2 $\mu\text{g/mL}$) was dispersed by sonication for 30 min. Then, the sheets were prepared by vacuum filtration of the dispersed solution onto a porous polycarbonate membrane whose diameter and pore size were 47 mm and 0.2 μm , respectively (Advantec, Japan). The sheets were fixed on a cell culture polystyrene dish (Culture PS; tissue-culture-treated, No. 430166, Corning), dried at 60°C for 3 h, and then sterilized under ultraviolet (UV) irradiation for 24 h.

2.2 Cells

Saos-2, a human osteosarcoma cell line, was obtained from Riken cell bank (Tsukuba, Japan). The human osteoblast-like cells (Saos-2) have been widely used as a model system for human osteoblastic cells in biomaterial studies. Cells were grown at 37°C in 5% CO_2 in Dulbecco's Modified Eagle's Medium (DMEM; Sigma) supplemented with 10% FBS (Biowest) and 1% PSN Antibiotic Mixture (penicillin-streptomycin-neomycin; Invitrogen, Carlsbad, CA, USA). Upon 70% confluence, the cells were detached, counted using a hemocytometer, and seeded on the CNT sheets.

2.3 Cell Adhesion Test

To estimate the kinetics of cell adhesion on CNT sheets, we carried out a cell adhesion test. The

1 sheets were pre-coated in DMEM containing 10% FBS and 1% PSN Antibiotic Mixture at 37°C in a
2 humidified 5% CO₂/95% air atmosphere for 1h. After the sheets were washed 3 times with
3 phosphate-buffered saline (PBS), osteoblast-like cells (Saos-2) were seeded at a density of 24,000
4 cells/cm² in DMEM containing 10% FBS, and incubated for 1, 3, or 6 h. For observation under
5 scanning electron microscope (SEM; S-4000, Hitachi, Japan), the sheets were rinsed with PBS to
6 remove the non-adhering cells, fixed with a 2.5% glutaraldehyde solution, and then dehydrated
7 following critical-point drying at 37°C. Cell adhesion was evaluated by counting the number of
8 attached cells on each sheet depicted in SEM images. Values representing the mean numbers and the
9 standard errors of the number of the attached cells were calculated from 10 different random fields
10 (480 × 600 μm²/field) of each sheet. Analysis of variance (ANOVA) and Student's *t* test were used to
11 assess the statistical significance of the results between groups. All statistical analyses were performed
12 at a confidence level of 95% by using a Microsoft Excel software.

13 To assess the spreading of the attached cells on the sheets, all the attached cells were divided
14 depending on their shape on SEM images: (A) round, cells are spherical in appearance as shown in Fig.
15 2b and 2e; (B) spread, cells extend their plasma membrane as shown in Fig. 2c and 2f.

16 To estimate the effect of pre-coating with serum, the sheets were incubated at 37°C under the

following conditions: (i) PBS for 1 h (control), (ii) 90% DMEM + 10% FBS for 1 h, and (iii) 90% DMEM + 10% FBS for 24 h. After the pre-coated sheets were washed 3 times with PBS, the cells were seeded at a density of 24,000 cells/cm² in DMEM containing 10% FBS and then incubated for 1 h. Cell adhesion was evaluated by a procedure similar to the one mentioned above.

3. Results and discussion

Fig. 1 shows the number of attached cells on the CNT sheets that was calculated using the cell adhesion test. At the initial stage, i.e., after 15 min, the number of attached cells on the sheets was small; this implies that a rapid adhesion such as an electrostatic adhesion did not occur for several minutes. Subsequently, the number of attached cells on the sheets increased considerably after 1-h incubation. Statistical analysis revealed that after 1 h, the number of attached cells on the MWNT sheets was greater than that on the SWNT sheets and Culture PS ($p < 0.05$). Further, the number of attached cells on the MWNT sheets seemed to be greater than that on the SWNT sheets and Culture PS after 3 h and 6 h (not significant, $p > 0.05$). These results indicate that MWNT sheets exhibit faster adhesion of cells. Moreover, the MWNT sheets exhibit both high speed and good capacity for cell

adhesion. The number of attached cells on the CNT sheets exhibited a peak after 1 h, but the number decreased slightly after 3 h. On the other hand, the number of cells on Culture PS gradually increased with time. These results regarding cell behavior that indicate a peak in the number of attached cells on CNT sheets are similar to those showing a peak in cell adhesion on a hydrophobic surface such as a polystyrene substrate [7]. Thus, the appearance of a peak of attached cells on CNT sheets at an initial stage can be attributed to their hydrophobic surface.

In Fig. 2a and 2d, the surface of CNT sheets was observed under an SEM. The surface of the SWNT sheets was comparatively smooth, while that of the MWNT sheets was rough and showed a three-dimensional network due to their low flexibility. Cell adhesion is known to be enhanced with an increase in the roughness of the surface within a limited range [8]. The attached cells were maximum on the rough surface of the MWNT sheets (see Fig. 1). Further, the number of filopodia that extended on the MWNT network (Fig. 2e and 2f) was greater than on the SWNT sheets (Fig. 2b and 2c). The SEM images of cell periphery on the MWNT sheets revealed several filopodia extending from the cells into the inner three-dimensional network, which was constructed with the MWNTs (detailed data not shown). Both the features of MWNT sheets, i.e., rough surface and the three-dimensional network of MWNTs probably provide a grip for cell adhesion [2,3]. These SEM results also suggest that the

MWNT sheets exhibit a high cell adhesiveness.

Next, the spreading of attached cells on the CNT sheets was assessed by enumerating round and spread cells by using an SEM (Fig. 3). The number of round cells or spread cells was significantly different on each substrate at various time points. The number of spread cells on all the substrates gradually increased with time. The number of spread cells on both Culture PS and the MWNT sheets was considerably higher than that on the SWNT sheets at all time points ($p < 0.05$, except in Culture PS vs. SWNT sheets after 1 h). On the other hand, the number of round cells on the MWNT and SWNT sheets was significantly higher than that on Culture PS at all time point ($p < 0.05$). An interesting observation was that most of the attached cells on Culture PS were of the “spread” type; the percentage of spread cells after 1, 3, and 6 h was 76%, 91%, and 95%, respectively. However, in the case of MWNT sheets, the percentage of spread cells after 1, 3, and 6 h was 55%, 71%, and 67%, respectively, which was lower than that in the case of Culture PS. At 6 h, Culture PS exhibited a large number and high percentage of spread cells, while the MWNT sheets exhibited a large number and low percentage of spread cells. These results indicate that the surface of the MWNT sheets facilitates cell adhesion but not the spreading of the attached cells. Other studies have also shown that osteoblastic

cells cultured on MWNTs there tend to acquire a spherical shape [9,10]. The rough surface of the MWNT sheets probably inhibits the spreading of the attached cells.

In order to estimate the effect of pre-coating on cell adhesion to CNT sheets, the sheets were pre-coated prior to the cell adhesion test. The conditions of pre-coating were as follows: PBS for 1 h (control), 90% DMEM + 10% FBS for 1 h, and 90% DMEM + 10% FBS for 24 h. As shown in Fig. 4, the number of attached cells on all the substrates was significantly different depending on the pre-coating condition. When the CNT sheets were pre-coated with 10% FBS for 1 h, the number of attached cells was the greatest ($p < 0.05$). With regard to cell adhesion to CNT sheets, pre-coating with PBS for 1 h and 10% FBS for 24 h showed a slight difference. On the other hand, the number of attached cells on Culture PS did not differ considerably among the conditions of pre-coating, and there was no significant difference in the numbers of attached cells among the pre-coating conditions ($p > 0.05$).

These results indicated that adhesion of osteoblast-like cells to CNT sheets could be greatly influenced by the pre-coating conditions. CNTs are known to exhibit high adsorption for various proteins, e.g., bovine serum albumin [11]. Casey *et al.* found that components of both the cell culture medium and the FBS growth supplement interact with CNTs, and their interactions affect cell

1 responses [4]. In addition, pre-coating with cell-adhesive proteins is known to improve the ability of
2 cell adhesion on cell culture substrates [12]. Thus, cell adhesion to CNT sheets is probably influenced
3 by protein adsorption on the substrate and is improved by the adsorption of cell-adhesive proteins from
4 the serum. However, proteins usually undergo a change in conformation including denaturation or
5 unfolding after adsorption on a substrate [13]. Despite sufficient protein adsorbing on the CNT sheets
6 pre-coated under 10% FBS for 24 h, the number of attached cells was lower; this could be due to
7 inactivation of cell-adhesive proteins such as fibronectin, laminin, and vitronectin, by their denaturation
8 or unfolding. The surface of Culture PS is known to be modified and exhibits moderate hydrophilic
9 cell adhesion property. However, it has a weak ability of protein adsorption (data not shown).
10 Therefore, cell adhesion to Culture PS could be influenced by the chemical composition to a large
11 extent than by their ability of protein adsorption. It can thus be speculated that cell adhesion to Culture
12 PS is not greatly influenced by pre-coating conditions

14 **4. Conclusions**

In this study, we investigated adhesion of human osteoblast-like cells (Saos-2) to SWNT and MWNT sheets. The MWNT sheets exhibited a faster adhesion of cells at an initial stage, i.e., after 1-h incubation than SWNT sheets and Culture PS. Moreover, the MWNT sheets demonstrated both high speed adhesion and good capacity for cell adhesion. However, the surface of the MWNT sheets facilitated cell adherence but not their spreading. Interestingly, cell adhesion to CNT sheets was significantly influenced by pre-coating with serum. CNT sheets exhibited a high cell adhesiveness after they were pre-coated under 10% FBS for 1 h. These results indicate that CNT sheets would play an important role in adsorption of serum proteins, which would consequently facilitate cell adhesion, and that MWNT sheets have a high cell adhesiveness.

CNT sheets can probably adsorb various proteins, including adhesion factors, growth factors, and differentiation-inducing factors. Thus, CNT sheets could be useful as a frame material, which can adjust its functions for cell culturing.

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Figure captions:

Fig. 1. The number of attached cells on the CNT sheets with respect to time.

The symbols indicate the following: ♦, Culture PS (control); ●, SWNT sheets; and □, MWNT sheets.

Error bars indicate a standard error for $n = 4$. Dotted line represents the number of the seeded cells. * $p < 0.05$.

Fig. 2. SEM images of the CNT sheets and a typical morphology of the attached cells on the sheets.

(a) SWNT sheets, (b) round cell on SWNT sheets, (c) spread cell on SWNT sheets, (d) MWNT sheets, (e) round cell on MWNT sheets, and (f) spread cell on MWNT sheets. White arrows indicate numerous filopodia extending from the cells.

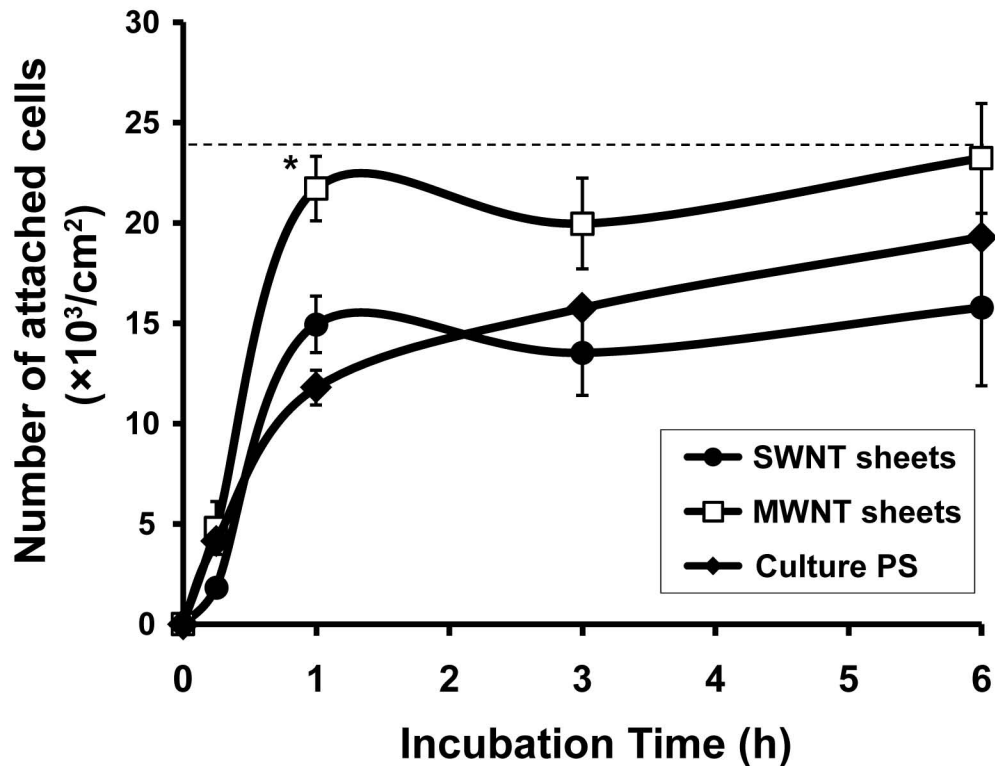
Fig. 3. The number of round and spread cells among attached cells on CNT sheets.

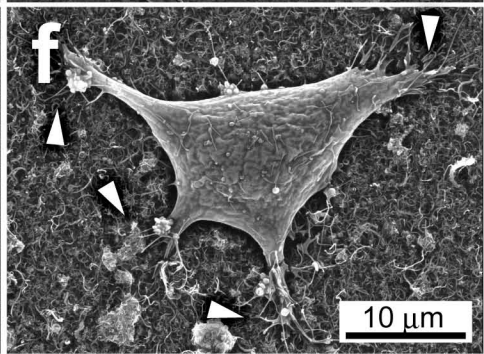
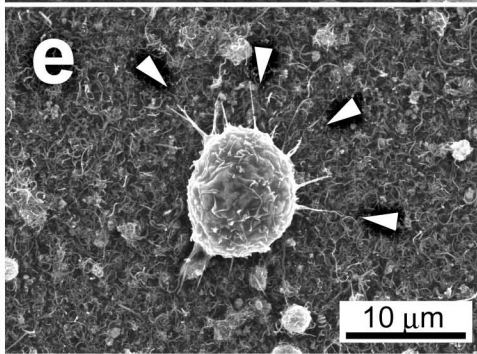
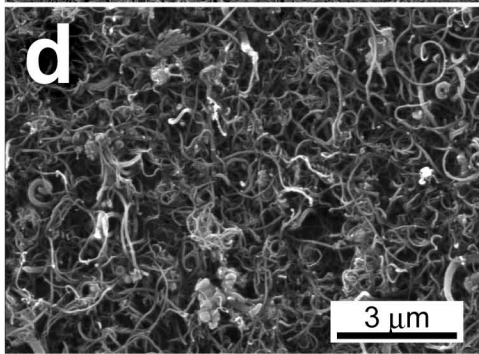
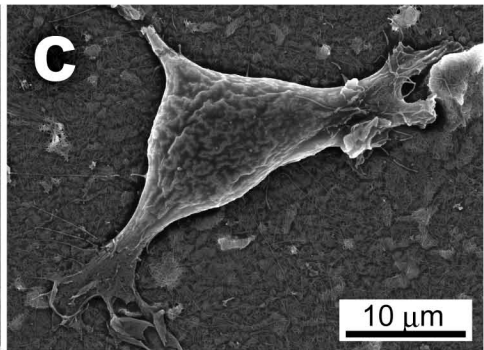
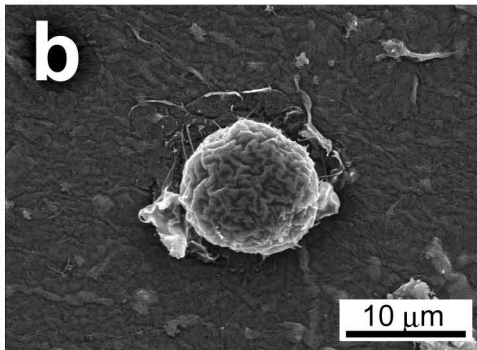
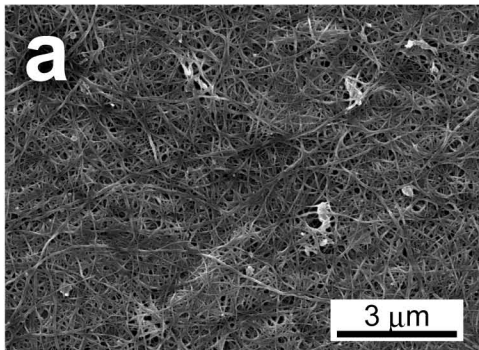
The number of round and spread cells among attached cells was measured on (a) Culture PS (control), (b) SWNT sheets, and (c) MWNT sheets. The gray and white columns indicate the number of round and spread cells, respectively. The error bars indicate a standard error for $n = 4$.

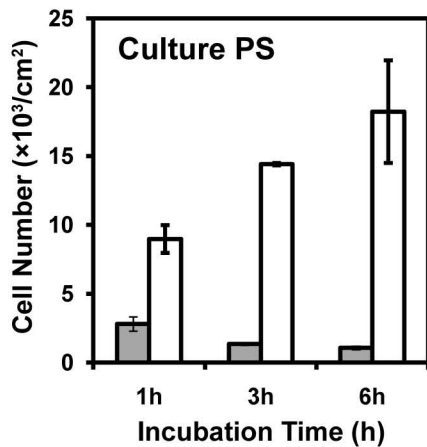
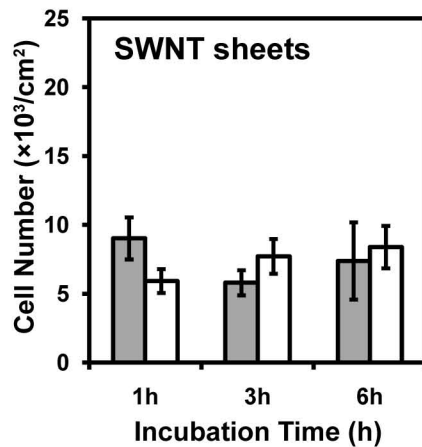
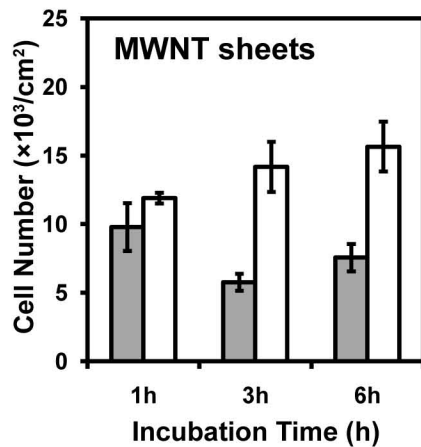
1

2 **Fig. 4.** Effects of pre-coating with serum on cell adhesion to CNT sheets.

3 The number of attached cells on Culture PS, SWNT sheets, and MWNT sheets was observed after 1-h
4 incubation, following pre-coating of the sheets under (white column) PBS for 1 h as a control, (gray
5 column) 10% FBS for 1 h, and (black column) 10% FBS for 24 h. The error bars indicate a standard
6 error for $n = 4$. * $p < 0.05$.





(a)**(b)****(c)**

Round Cell



Spread Cell

