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Strong Adhesion of Saos-2 Cells to Multi-walled Carbon Nanotubes

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Abstract

In recent years, carbon nanotubes (CNTs) have been considered potential biomedical materials because of their unique character. The aim of this study was to investigate the response of a human osteoblast-like cell line—Saos-2—on single-walled CNTs (SWCNTs) and multi-walled CNTs (MWCNTs). The surface of a culture dish was coated with CNTs, and Saos-2 cells were cultured for three days. Cell morphology, viability, alkaline phosphatase (ALP) activity, adhesion, and vinculin expression were evaluated. The result showed high cell viability and strong adhesion to MWCNTs. Saos-2 cultured on MWCNTs exhibited vinculin expression throughout the cell body, while the cells attached to SWCNTs and glass were mostly limited to their periphery. Our results suggest that CNT coatings promote cell activity and adhesiveness. These findings indicate that MWCNTs could be used as surface coating materials to promote cell adhesion.

Keywords: carbon nanotubes, osteoblast, adhesion, vinculin

1. Introduction

Carbon nanotubes (CNTs) constitute sheets rolled into cylinders with diameters in the nanometer range. They are composed of a single cylinder, i.e., a single-walled carbon nanotube (SWCNT), or multiple concentric cylinders, i.e., a multi-walled carbon nanotube (MWCNT). CNTs have attracted much attention across various fields such as electrical, chemical, and mechanical fields [1–4]. They are also considered potential biomedical materials because of their nanoscale dimensions, high strength, flexible structure, and their electrical conductivity [5–8].

In the field of tissue regeneration, several studies have been conducted the reaction of cells to the nanoscaled topography of CNT-based scaffolds. Zhang *et al.* [9] reported that cell viability and distribution of focal adhesion kinases (FAKs) differed between the cells cultured on SWCNTs and MWCNTs. Firkowska *et al.* [10] reported on the differences between osteoblast adhesion on glass and MWCNT-based substrates with respect to vinculin expression patterns and Young's modulus; this study indicated that cellular behaviors were influenced even by nanoscaled dimensions.

In this study, we investigated the response of Saos-2, a human osteoblast-like cell line, on SWCNTs and MWCNTs. To examine the impact of the CNT substrate, cell morphology, cell viability, and alkaline phosphatase (ALP) activity were studied. Cell adhesion to the substrate was evaluated on the basis of adherent cell number after the trypsin–EDTA treatment. Vinculin expression was also visualized to examine the relation between cell adhesion and the substrate.

2. Experimental procedure

2.1. Preparation of CNT-coated dish

Two types of CNTs—SWCNTs and MWCNTs—were used in this study. The SWCNTs, synthesized using the arc discharge method, were 0.8–2.5 nm in diameter and had a purity of >95% (Meijo Nano Carbon Co., Ltd., Japan) [11]. The MWCNTs, synthesized using the chemical vapor deposition technique (NanoLab Inc., MA, USA), were 20–40 nm in diameter and had a purity of 98% [12]. Both types of CNTs were dispersed in 99.5% ethanol by sonication. Fifty microliter of the resultant dispersion was poured onto a culture dish (Corning, NY, USA) and dried at room temperature; this process was repeated until the quantity of CNTs on the surface of the dish reached $0.5 \mu\text{g}/\text{cm}^2$. Thus culture dishes were prepared in 2 ways: SWCNT-coated dish and MWCNT-coated dish. All dishes were sterilized by ultraviolet radiation for 24 h prior to experiments with cells.

2.2. Surface observation of CNT-coated dish

The surface morphology of the 2 types of substrates was characterized using a scanning electron microscope (SEM; Hitachi S-4000; Hitachi, Japan). Samples were sputter-coated with platinum-palladium and all micrographs were recorded at 10 kV.

2.3. Cell culture

All experiments were performed using Saos-2, the human osteoblast-like cell line. Cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM; Sigma

Aldrich, USA), supplemented with 10% fetal bovine serum (FBS; MP Biomedicals, Germany) under standard cell culture conditions (at 37°C in a humidified atmosphere with 5% CO₂). The medium was replaced every 2 days.

2.4. Cell morphology

Saos-2 cells cultured on the SWCNT- and MWCNT- coated dish for three days were fixed with 2.5% glutaraldehyde and dehydrated with increasing concentrations of ethanol (50%, 70%, 80%, 90%, 95%, and 100%), and dried at critical-point. The samples were then coated with a thin layer of platinum-palladium and observed under a SEM.

2.5. Cell viability

Cell viability was determined using the Alamar Blue assay [13]. Cells were seeded onto the SWCNT-coated dish, MWCNT-coated dish, and culture dish at a density of 2.5×10^4 cells/ml. After cultivation for three days, the cells were rinsed with phosphate-buffered saline (PBS); thereafter 2 mL of fresh medium containing 5% Alamar blue (Biosource, USA) was added to each dish. Following 3 h incubation with the dye, absorbance was measured at 570 nm and 600 nm using a spectrophotometer (U-1100, Hitachi, Japan). Cell viability was normalized with DNA content. The DNA content of the cells on each dish was measured using PicoGreen (Molecular Probes, Leiden, Netherlands), according to the manufacturer's protocol. The cells cultured on the culture dish were used as controls (100% viability).

2.6. Alkaline phosphatase (ALP) activity

Cells were seeded onto each of 3 dishes at a density of 2.5×10^4 cells/ml. After cultivation for three days, the culture medium was removed, the cells were rinsed with PBS twice, and 1 mL of the lysate buffer was added. Cells on the substrate were scraped and homogenized using sonication for 2 min. ALP activity was measured by using a LabAssay ALP kit (Wako, Japan) according to the manufacturer's protocol. ALP activity was normalized with DNA content. The DNA content of the cells on each dish was measured using PicoGreen, according to the manufacturer's protocol.

2.7. Cell adhesion test

The adhesive strength of the Saos-2 cells to the CNT-coated dish was tested using diluted trypsin–EDTA solution (Gibco, USA). Cells were seeded onto the SWCNT-coated, MWCNT-coated, and culture dishes at a density of 1.2×10^4 cells/ml. After cultivation for three days, the cells were washed with PBS twice and treated with 0.1% trypsin–EDTA solution for 5 min, 10 min, 20 min, and 30 min at 37°C in a humidified atmosphere with 5% CO₂. After the treatment, each substrate was washed twice with PBS to remove the detached cells. The cells that remained adherent to the dish were fixed with 2.5% glutaraldehyde and stained with 4',6'-diamidino-2-phenylindole (DAPI, Merck). Twenty photographs of each substrate were acquired and the cell numbers were counted. Measurements were performed in 3 samples each. The adhesive strength was evaluated using the ratio of the adherent cell number after the trypsin–EDTA treatment to the untreated cell number (% control).

To evaluate the relation between culture period and adhesive strength, the cells

cultured for one or three days on each substrate were treated with 0.1% trypsin–EDTA solution for 10 min and the adherent cell percentages were compared between culture period.

2.8. Immunofluorescence staining

For the visualization of the vinculins, Saos-2 were seeded on the slide glass, SWCNT-coated glass ($0.5\mu\text{g}/\text{cm}^2$) and MWCNT-coated glass ($0.5\mu\text{g}/\text{cm}^2$) for three days, washed with phosphate buffer saline (PBS), and fixed with 3.7% formaldehyde in PBS for 15 min. Thereafter, the cells were permeabilized with 0.1% Triton X-100 for 20 min and, blocked with 1% bovine serum albumin in PBS for 30 min. Vinculins were visualized by using a Subcellular Structure Localization Kit (Millipore, USA), according to the manufacturer's protocol. Briefly, cells were incubated with a 1:100 dilution of mouse anti-human vinculin for 1 h at room temperature. Subsequently, the cells were labeled with Goat anti-Mouse IgG, FITC conjugated secondary antibody. For nuclei staining, cells were incubated with DAPI at 37°C in a humidified atmosphere for 45 min. Cells were visualized using a fluorescence microscope (BZ9000, Keyence, Japan).

2.9. Statistical analysis

Analysis of variance (ANOVA) and Student's *t*-test were used to assess the statistical significance of the results between groups. Values of $p < 0.05$ were considered to be significant.

3. Result

Figure 1 shows the SEM micrographs of the surface of CNT-coated dishes and the Saos-2 cells cultured for three days on CNTs. The surface of the culture dishes was coated with either SWCNTs (Fig. 1a) or MWCNTs (Fig. 1b). The SWCNT coating exhibited a flat and net-like pattern, while the MWCNTs coating exhibited a shorter, curled structure. SEM examinations of Saos-2 cells revealed that the cells were extended in a similar pattern on both the SWCNTs (Fig. 1c) and MWCNTs (Fig. 1d), and that there was no obvious difference between the patterns.

The viability of cells cultured on the culture dish, SWCNTs and MWCNTs were shown in Figure 2a. The viability of the cells cultured in the MWCNT-coated dish ($156 \pm 9\%$) was significantly higher than that of cells cultured in the culture dish. The cells cultured in the SWCNT-coated dish showed higher viability ($130 \pm 8\%$) than the cells cultured in the culture dish, but the difference was not a statistically significant level. Figure 2b represents the ALP activity at three days of culture on each dish. As for the ALP activity, there was no significant difference among the three types of dishes. Thus, the ALP activity of Saos2 cells was not affected by direct contact with CNTs.

Figure 3a shows the ratio of the adherent cell number after 5–30 min of treatment with 0.1% trypsin–EDTA. As shown in the graph, the ratio of the adherent cell number decreased with the increase in treatment time in all substrates. Almost all the cells cultured on the culture dish and SWCNT dish detached within 10 min of treatment. In contrast, approximately 30% of cells cultured on the MWCNT dish remained after 10 min of treatment and more than 10% of cells remained even after 30 min of treatment. To evaluate the relation between culture period and adhesive strength,

cells cultured for one or three days on each substrate were compared. Figure 3b shows the ratio of the adherent cell number after 10 min of treatment with 0.1% trypsin–EDTA. Most cells cultured on the culture dish and the SWCNT dish detached after treatment at both periods, while the cells cultured on the MWCNT dish showed a higher adherent cell ratio at both periods. Moreover, the adherent cell ratio on culture day 3 was significantly (approximately 10%) higher than that on day one.

Figure 4 shows the vinculin expression of Saos-2 cells cultured on the slide glass, SWCNT-coated glass, and MWCNT-coated glass for three days. The distribution pattern of vinculin differed between cells cultured on each substrate. Cells on the slide glass (Fig. 4a) and the SWCNT-coated glass (Fig. 4b) expressed vinculin that was primarily limited to the peripheral part of the cell body; in contrast, the cells cultured on the MWCNT-coated glass (Fig. 4c) exhibited vinculin expression throughout the cell body.

4. Discussion

We investigated the impact of SWCNTs and MWCNTs on Saos-2, a human osteoblast-like cell line. Both types of carbon nanotubes were dispersed in ethanol using sonication without any modification, in order to ensure that the influence of the CNT itself could be evaluated.

The SEM image (Fig. 1c and 1d) shows the morphology of Saos-2 on SWCNTs and MWCNTs. The cell body was flat and well spread on both substrates.

Cells cultured on the CNT-coated dish showed higher viability than those on the culture dish (Fig. 2a). The ALP activity levels of Saos-2 on CNTs were similar to

those on the culture dish (Fig. 2b). Wirth *et al.* [14] reported the behavior of rat osteoblasts on NiTi with different surface roughness. They showed that surface roughness did not influence ALP activity, although it stimulated cell activity. This indicates that the higher viability of Saos-2 on the MWCNTs might be attributable to the difference in the surface structure (Fig. 1a and 1b). From the results of cell morphology, viability, and ALP activity, no obvious cytotoxic effect of CNTs was demonstrated in this experimental period.

The cell adhesion strength to the substrate was evaluated using the adherent cell ratio after trypsin–EDTA treatment. Compared to those cultured on the SWCNTs and culture dish, the cells cultured on the MWCNT-coated dish showed significantly strong adhesions. After 10 min of treatment, more than 20% of cells remained adherent onto the MWCNT-coated dish, although almost all cells on the SWCNT-coated dish and culture dish detached (Fig. 3a). To investigate the mechanism of the difference, vinculin expression on the slide glass and CNT-coated glass was visualized. From the result of the immunofluorescence staining, the difference in adhesion strength was explained by the difference in focal adhesion. Saos-2 cells cultured on MWCNTs exhibited vinculin expression throughout the cell body (Fig. 4c), while cells on the slide glass and SWCNTs showed vinculin expression in a limited area (Fig. 4a and 4b). These findings suggested that the rough and curled nanostructure of MWCNTs stimulated the vinculin expression of Saos-2 and contributed to the strong adhesion. The results of one and three day cultivation (Fig. 3b) indicated that the adhesive strength increases with the culture day. The results also suggested that the increase in adhesion strength might be caused by focal adhesion, not because of mere attachment to a projection of

MWCNTs. Moreover, the comparison of cell adhesion strength between SWCNTs and MWCNTs indicates that altered vinculin expression was not the result of the chemical composition. From these experiments, we concluded that strong adhesion to the MWCNT-coated dish was attributable to the rough and curled structure of MWCNTs.

5. Conclusion

In this study, MWCNT- and SWCNT-coated dishes were used as substrates and the cell morphology, viability, ALP activity, adhesion strength, and vinculin expression levels of Saos-2 cells were evaluated. The results of cell morphology and ALP activity examinations suggested that Saos-2 cells had good affinity towards both types of CNTs. In particular, cells cultured on MWCNTs showed high viability and adhesiveness. The vinculin expression pattern on MWCNTs was different than that of other substrates; we believe that this was because of the topography of the surface. On the basis of these results, it can be concluded that both types of CNTs could be used as biomedical materials, and that MWCNTs should particularly be used as surface coating materials to promote cell adhesion.

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Figures

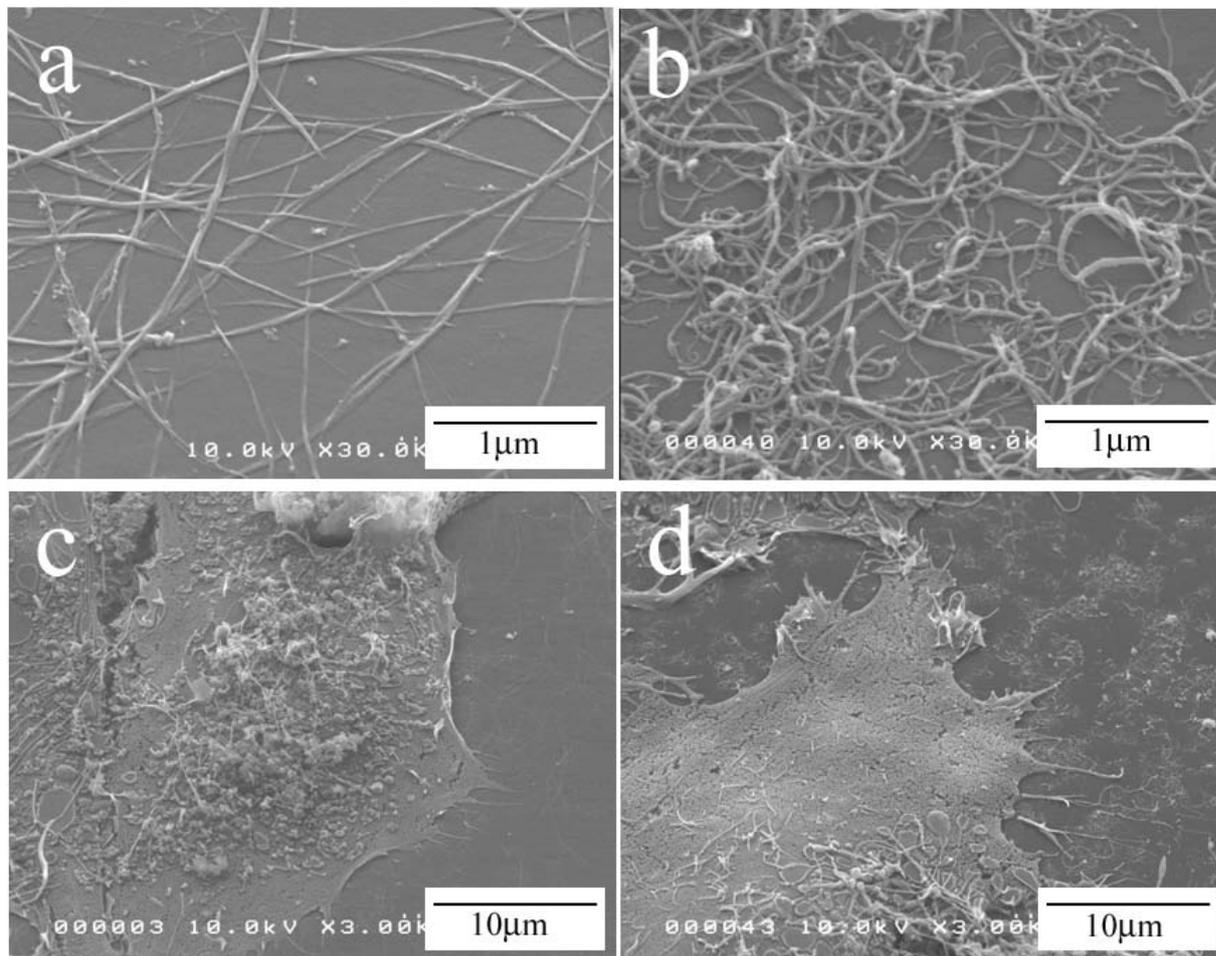


Fig. 1. Scanning electron microscope (SEM) images of the surface structure of SWCNT-coated dish (a), MWCNT-coated dish (b). SEM images of Saos-2 cells cultured for three days on SWCNTs (c) and MWCNTs (d).

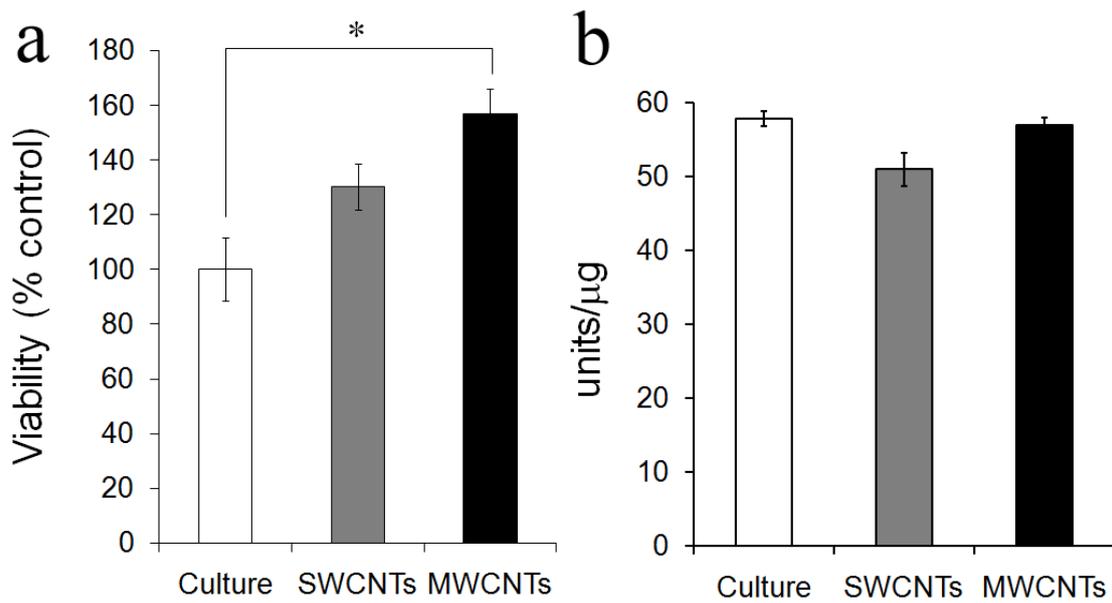


Fig. 2. Viability of Saos-2 cells cultured on Culture, SWCNT and MWCNT dishes for three days (a). Cells on culture dish were used as controls (100% viability). ALP activity of Saos-2 cells on the culture dish, SWCNTs, and MWCNTs at three days (b). Both Viability and ALP activity were normalized to the DNA content. The data are expressed as mean \pm SE (n = 3). *p < 0.05.

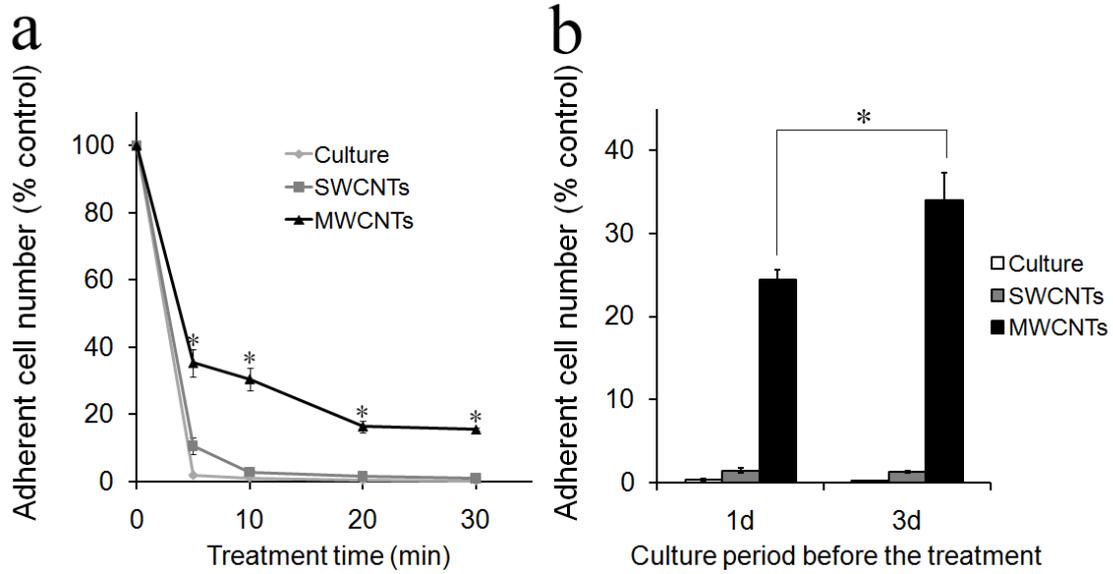


Fig. 3. The ratio of the adherent cell number after 5–30 min of 0.1% trypsin–EDTA treatment (a) The data are expressed as mean \pm SE (n = 3). *p < 0.05 between control (Culture). To evaluate the relation between culture period and adhesive strength, ratio of adherent cells cultured for one or three days on each substrate were compared (b). The graph shows the ratio of the adherent cell number after 10 min of treatment with 0.1% trypsin–EDTA. The cell number without trypsin–EDTA treatment was used as a control. The data are expressed as mean \pm SE (n = 3). *p < 0.05 between culture periods.

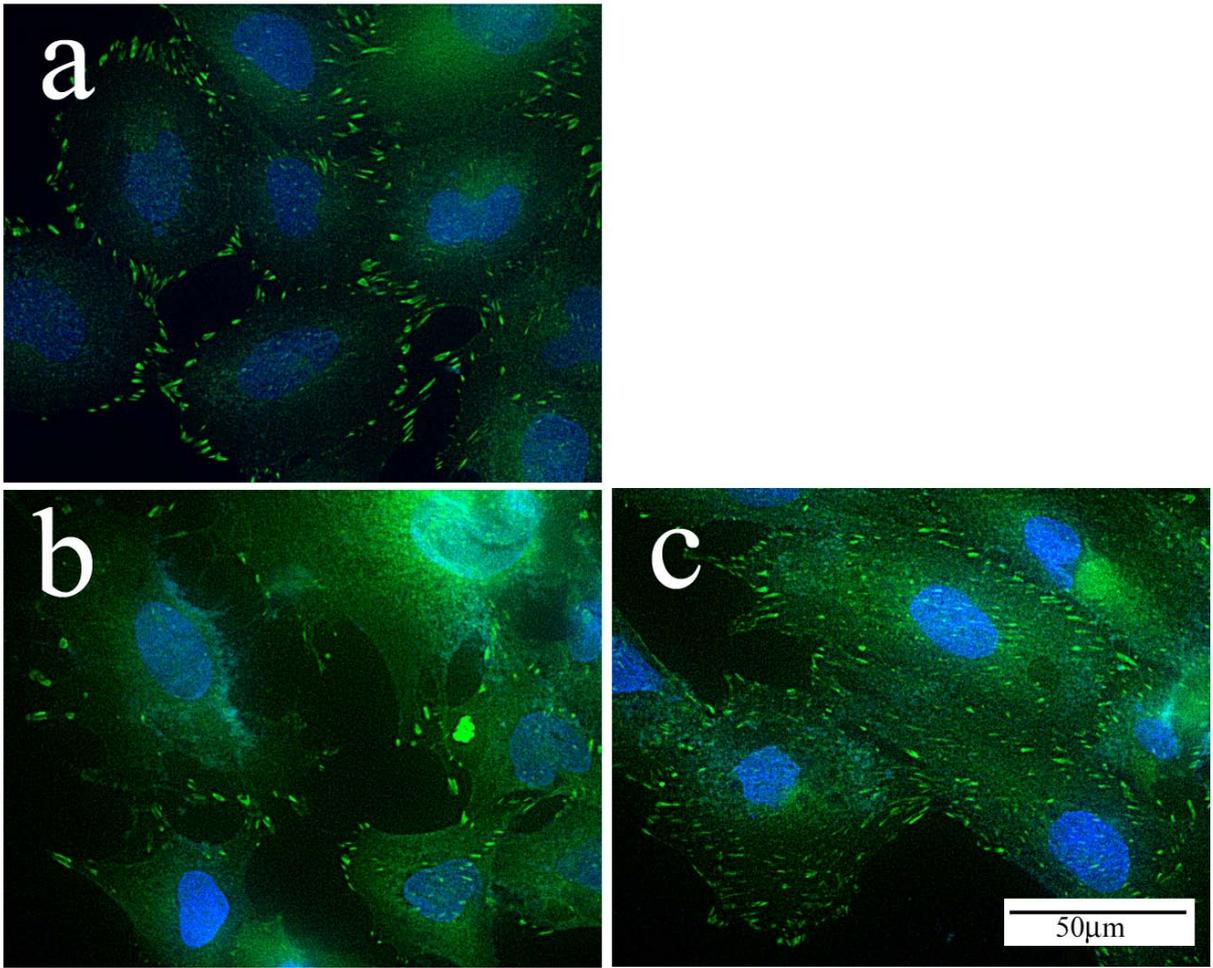


Fig. 4. Vinculin expression patterns of Saos-2 cells cultured on the slide glass (a), SWCNT-coated glass (b), and MWCNT-coated glass (c) for 3 days.