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Citation	Materials Science and Engineering: C, 30(3), 391-399 https://doi.org/10.1016/j.msec.2009.12.006
Issue Date	2010-04
Doc URL	http://hdl.handle.net/2115/48563
Type	article (author version)
File Information	Akasaka20120314_2.pdf



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1 **Thin films of single-walled carbon nanotubes promote human osteoblastic**
2 **cells (Saos-2) proliferation in low serum concentrations**

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15 Keywords: Carbon nanotubes; Thin film; Cell proliferation; Serum; Osteoblastic cells;
16 Protein adsorption

17

1 **Abstract**

2

3 One strategy used for the regeneration of bone is the development of cell culture substrates
4 and scaffolds that can control osteoblast proliferation and differentiation. In recent
5 investigations, carbon nanotubes (CNTs) have been utilized as scaffolds for osteoblastic cell
6 cultures; however, there are only a few reports describing the proliferation of osteoblastic
7 cells on thin CNT films; in particular, the effects of serum concentration on cell proliferation
8 has not been studied. In the present study, we prepared culture dishes with homogeneous thin
9 or thick films of non-modified CNTs and examined the effect of serum concentrations on
10 human osteoblastic cells (Saos-2) proliferation in these culture dishes. We demonstrated that
11 the ratio of cell proliferation was strongly affected by the concentration of serum.
12 Interestingly, single-walled carbon nanotube (SWNT) thin films were found to be the most
13 effective substrate for the proliferation of Saos-2 cells in low concentrations of serum. Thus,
14 thin SWNT films may be used as an effective biomaterial for the culture of Saos-2 cells in
15 low serum concentrations.

16

17

1 **1. Introduction**

2

3 Carbon nanotubes (CNTs) have attracted considerable attention because of their unique
4 physical properties and their potential use in a variety of applications. CNTs are structural
5 carbons having 2 structural forms: single-walled carbon nanotubes (SWNTs) and
6 multi-walled carbon nanotubes (MWNTs). SWNTs can be considered as long wrapped
7 graphene sheets with diameters typically ranging from 0.5 to 1.5 nm. MWNTs have larger
8 diameters typically ranging from 10 to 100 nm owing to their multilayered structures. In
9 recent investigations regarding biological applications [1], CNTs have been utilized as
10 scaffolds for cell cultures [2–6]. Mattson *et al* reported the first application of CNTs as a
11 scaffold for the growth of nerve cells [7]. In addition, the use of chemically functionalized
12 CNTs as a substrate allows for the control of the outgrowth and branching patterns of
13 neuronal processes [8,9]. Sheets of CNTs can also be used as a suitable scaffold for excellent
14 adhesion and proliferation of osteoblastic cells, bone formation by osteoblastic cells [10,11],
15 and for the successful differentiation of neural stem cells [12]. Furthermore, protein
16 adsorption by CNT scaffolds from the serum in cell culture medium might enhance cell
17 adhesion, proliferation, and growth [11,13–15]. The functions of CNT scaffolds may be
18 greatly influenced by the concentration of serum in cell culture medium.

19 The field of regenerative medicine has been developed with the aim of regenerating and

1 remodeling tissues *in vivo* for repairing, replacing, maintaining, or enhancing the functions of
2 tissues and organs [16,17]. In one of the approaches used in regenerative medicine, a patient's
3 cells can be proliferated *in vitro*, and then these cells are implanted back into the patient's
4 damaged organ or tissues to avoid the induction of an immune rejection response. When a
5 patient's cells are cultured in the patient's own serum *in vitro*, the likelihood of induction of
6 an immune rejection response is less. In this case, cell culture substrates for the effective
7 proliferation of cells in low concentrations of serum are required to decrease the amount of
8 patient's serum used for culture. Other advantages of such cell culture substrates, which are
9 attributed to the decreased amount of serum used, are their use as biomaterials to enable easy
10 control of cell differentiation, to simplify purification of cell products, to enable effective
11 transfection into cells, to provide high sensitivity for the detection of toxicity in *in vitro*
12 toxicity assays, and so on [18–20].

13 Bone injuries occur due to various reasons, including degenerative, surgical, and traumatic
14 processes. One strategy used for the regeneration of bone is the development of cell culture
15 substrates and scaffolds that can control the osteoblast proliferation and differentiation [21].
16 In our recent studies, we demonstrated the excellent properties of CNT sheets for their use as
17 scaffolds for the culture of osteoblastic cells [11,14,15]; however, there are only a few reports
18 describing the proliferation of osteoblastic cells on thin films of CNTs, and the effects of
19 serum concentration on cell proliferation have not been studied.

1 To investigate the types of CNTs that can most effectively promote cell proliferation in low
2 concentrations of serum, we cultured human osteoblast-like cells (Saos-2) on CNT scaffolds.
3 Saos-2 cells have been widely used as a model system for human osteoblastic cells in
4 biomaterial studies for tissue engineering. In the present study, we prepared CNT-coated
5 dishes with homogeneous thin or thick films by coating non-modified CNTs on the surface of
6 a commercially available non-treated polystyrene dish (Normal PS). The resulting transparent
7 films of CNTs, with different CNT types and thicknesses were assessed by the morphology of
8 the cells cultured on them. Then, we characterized Saos-2 cells proliferation on both thin and
9 thick CNT films in 1–20% serum concentration. This study is the first to report that thin films
10 of SWNTs can most effectively promote osteoblastic cells (Saos-2) proliferation in low
11 concentrations of serum.

12

13

14 **2. Experiments**

15

16 *2.1. Preparation of CNT-coated dishes*

17

18 Purified SWNTs (0.8–2.5 nm in diameter) were purchased from Meijo Nano Carbon Co., Ltd.
19 (Nagoya, Japan) and synthesized by the arc discharge method [22]. The MWNTs (average

1 diameter of 30 nm) were purchased from NanoLab Inc. (Brighton, MA, USA) and were
2 purified using a previously reported method [23]. The SWNTs had a purity of >95 wt% and
3 contained <5 wt% amorphous carbon as the dominant impurity, while the MWNTs had a
4 purity of >98 wt% and contained <2 wt% amorphous carbon. To prepare the homogeneous
5 CNT coating, a dilute solution of CNTs in 99.5% ethanol (5 $\mu\text{g/ml}$ for SWNTs and 10 $\mu\text{g/ml}$
6 for MWNTs) was dispersed by ultrasonication. A 50- μl aliquot of the dispersed CNTs was
7 immediately spotted onto a 60-mm non-treated polystyrene dish (Normal PS), which has a
8 low adhesive surface for suspension culture in order to decrease the influence of the base
9 material layer. They were then dried at room temperature under a humidity of <30%. This
10 procedure was repeated until the necessary amount of CNT coating was obtained (Fig. 1).
11 The dishes were dried at 60°C for 3 h and sterilized by UV irradiation (DM-5; Daishin Co.,
12 Ltd., Osaka, Japan) for 1 day.

13 The following abbreviations have been used in this paper for the CNT-coated dishes:
14 SWNT-coated dishes, SWNT0.5 (0.5 $\mu\text{g/cm}^2$) and SWNT5 (5 $\mu\text{g/cm}^2$); MWNT-coated dishes,
15 MWNT0.5 (0.5 $\mu\text{g/cm}^2$) and MWNT5 (5 $\mu\text{g/cm}^2$). For comparison, we used commercially
16 available 60-mm-diameter polystyrene dishes as follows: non-treated polystyrene dish
17 (Normal PS; Corning Inc., NY, USA), cell culture polystyrene dish (Culture PS; Corning Inc.,
18 NY, USA), collagen-coated polystyrene dish (Collagen PS; Iwaki Co., Ltd., Tokyo, Japan),
19 and poly-L-lysine-coated polystyrene dish (Poly-Lys PS; Iwaki Co., Ltd., Tokyo, Japan).

1

2 *2.2. Surface roughness and topography*

3

4 To evaluate surface roughness, the CNT-coated dishes were characterized by using atomic
5 force microscopy (AFM). AFM was performed using a commercial Nanoscope IIIa (Veeco
6 Instruments Inc., Santa Barbara, CA, USA) in the tapping mode across an area ($2\ \mu\text{m} \times 2$
7 μm) of the sample surface using a silicon cantilever (Tap300 Metrology probe; MRP-11100,
8 Veeco Instruments Inc., Woodbury, NY, USA) in air under ambient laboratory conditions
9 (25°C). WSxM scanning probe microscopy software by Nanotec Electronica S.L. (Tres
10 Cantos, Madrid, Spain) and Gwyddion data analysis software covered by the GNU general
11 public license were used for AFM image analysis. The roughness of the surface was assessed
12 by measuring the roughness parameter, R_a (roughness average). Results are presented as
13 mean \pm standard error of 3 experiments. Scanning electron microscope (SEM) images were
14 using on a Hitachi S-4000 (Hitachi, Japan).

15

16 *2.3. Morphology of cells cultured on CNT-coated dishes*

17

18 Saos-2 cells were seeded at a low density ($1000\ \text{cells}/\text{cm}^2$) onto 60-mm CNT-coated dishes.
19 The cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Sigma-Aldrich

1 Co. Ltd., St. Louis, MO, USA) with 10% fetal bovine serum (FBS) (CELLect, Lot No.
2 3929H; MP Biomedicals, Inc., OH, USA) and 1% penicillin-streptomycin-neomycin (PSN)
3 Antibiotic Mixture (Invitrogen, Carlsbad, CA, USA) at 37°C in a humidified 5% CO₂/95%
4 air environment for 5 days. For optical microscope observations, the cells were stained by
5 Giemsa staining performed according to the general protocol. The stained cells were
6 visualized using the Nikon ECLIPSE TS100 microscope (Nikon Co., Ltd., Tokyo, Japan) and
7 digital images were acquired using a Nikon DS-5M-L1 camera (Nikon Co., Ltd., Tokyo,
8 Japan). For SEM observations, the dishes were washed with phosphate-buffered saline (PBS)
9 to remove the medium, fixed with a solution of 2.5% glutaraldehyde, and post-fixed in 1%
10 osmium tetroxide. The dishes were then dehydrated in a graded series of alcohol (50%, 70%,
11 80%, 90%, 95%, and 100%) following critical point drying at 37°C. SEM images were
12 obtained using a Hitachi S-4000.

13

14 *2.4. Effect of FBS concentration on cell proliferation*

15

16 Saos-2 cells were seeded at a low density (1000 cells/cm²) onto 60-mm CNT-coated dishes.
17 The cells were then cultured in DMEM with 1%, 10%, and 20% FBS (CELLect) and 1%
18 PSN Antibiotic Mixture at 37°C in a humidified 5% CO₂/95% air environment. The medium
19 was replaced every 2 days. After the cells were cultured to approximately 40–50% confluence

1 on the dish that showed the highest proliferation rate at each FBS concentration, the dishes
2 were washed with PBS, fixed with a solution of 2.5% glutaraldehyde, and stained by Giemsa
3 staining. Cell proliferation was evaluated from optical microscope images by counting the
4 number of cells attached to each dish. Values represent the mean number and standard errors
5 of spread cells calculated from 20 different random fields ($2.2 \times 1.6 \text{ mm}^2/\text{field}$) of each dish.
6 Analysis of variance (ANOVA) and Student's *t*-test were used to assess the statistical
7 significance of the results. All statistical analyses were performed with Microsoft Excel
8 software at a confidence level of 95%.

9

10 *2.5. Protein adsorption on the surface of CNT-coated dishes*

11

12 To evaluate protein adsorption by using an adsorption assay of fluorescent-labeled protein,
13 we added 4 ml of 1 $\mu\text{g/ml}$ PBS (pH 7.4) containing fluorescein isothiocyanate (FITC)-labeled
14 bovine serum albumin (FITC-BSA; Molecular Probes, Eugene, OR, USA) to 60-mm
15 CNT-coated dishes. After 10, 30, 60, and 120 min of incubation at 25°C, the fluorescence
16 intensity (Ex = 495 nm, Em = 520 nm) of the supernatant was measured using a fluorescence
17 spectrophotometer (F-2500; Hitachi, Japan). The initial value of fluorescence intensity was
18 set as 100% at 0 min incubation.

19

1 *2.6. Wettability of the surface before and after immersion in FBS*

2

3 For immersion in FBS, 4 ml DMEM with 1% FBS was added to the dishes. After 3 h
4 incubation at 25°C, the medium was removed and washed 5 times with ultra-pure water. The
5 dishes were dried at 60°C for 3 h and the surface wettability of the dishes was evaluated.

6 Ultra pure water (Kanto Chemical Co., Inc., Tokyo, Japan) drops (20 µl) were placed on the
7 surface of the dishes in an ambient environment. Contact angles were measured on a digital
8 microscope (VH-6300; Keyence, Osaka, Japan). Images of water spreading were recorded
9 with a CCD camera after 10 s and saved onto a PC, and automated contact angle
10 measurements were performed using ImageJ plug-in LB-ADSA [24].

11

12

13 **3. Results**

14

15 *3.1. Surface roughness and topography of CNT-coated dishes*

16

17 To evaluate surface roughness, the CNT-coated dishes were characterized by AFM. The
18 surface roughness (R_a) of CNT-coated dishes is shown in Table 1. The surface roughness of
19 the SWNT0.5 and SWNT5 dishes was 7.1 ± 0.6 nm and 15.8 ± 0.9 nm, respectively. The

1 surface roughness of the MWNT0.5 and MWNT5 surface was 7.9 ± 1.6 nm and 45.0 ± 1.7
2 nm, respectively. The R_a values of the thin SWNT0.5 and MWNT0.5 films tended to be
3 similar. In the same types of CNTs, the R_a values became higher with increasing amounts of
4 CNT coating. The surface of the thick MWNT5 films was rougher than that of the SWNT5
5 films. Figs. 2a and 2b show the AFM images ($2 \mu\text{m} \times 2 \mu\text{m}$) of the thick films. These images
6 show significant differences in roughness; however, all the R_a values were in the narrow
7 range of 7–45 nm. Thus, CNT coating can form a rough surface at the nano level, but only to
8 a limited extent.

9 To evaluate the surface topography, we used a SEM for observing the surface of the
10 CNT-coated dishes. Fig. 3 shows the SEM images of the surface of the dishes. There were
11 slight differences in the fibrous morphology of SWNT and MWNT-coated dishes. The
12 SWNTs were arranged into a straight line of bundles with a diameter of 10–100 nm. In
13 contrast, the MWNTs were arranged into curved line with a diameter of 20–100 nm. The
14 diameter of the bundles of SWNTs was equivalent to the diameter of MWNTs. The surface of
15 thin films (SWNT0.5 and MWNT0.5) in the concentration of $0.5 \mu\text{g}/\text{cm}^2$ was not completely
16 covered with CNTs; hence, a large area of the surface of the base substrate was exposed. As
17 shown in Figs. 3a and 3c, SWNT0.5 films formed a fine network, while MWNT0.5 films
18 formed a coated pattern on the surface of the dish, respectively. Compared to these dishes, in
19 the SWNT5 and MWNT5 dishes, the CNTs formed a densely packed mesh work

1 nanostructure that covered the entire surface. The surface of the base substrate itself was
2 slightly exposed between the bundles of the SWNTs (Fig. 3b) or the fibrous lines of the
3 MWNT network (Fig. 3d). These AFM and SEM observations reveal that the SWNT coating
4 yielded a flat surface that could be attributed to their softness, while the MWNT coating
5 yielded a three-dimensional network that could be attributed to their hardness.

6 Kalbacova *et al* reported that SWNT films prepared without using surfactants, minimized
7 the effect of other agents on the behavior of monocytes/macrophages and osteoblastic cells
8 [25,26]. In addition, the advantages of ethanol as a coating solvent are its high volatility and
9 adequate dispersion of CNTs in a dilute solvent solution by ultrasonication. Furthermore, in
10 our method, using a small amount of ethanol allows the attachment of the dispersed CNTs to
11 the dish surface faster than the reaggregation of the dispersed CNTs, which could be
12 attributed to the fast evaporation process. Therefore, thin films with few aggregated CNTs
13 can be formed with non-modified CNTs. If repeated coating is performed until the necessary
14 amount of CNT coating is obtained, thicker films can be made with fewer aggregated CNTs.

15

16 *3.2. Morphology of cells cultured on CNT-coated dishes*

17

18 To evaluate the effects of the amount and type of CNT films on the morphology of cells, the
19 morphology of osteoblastic cells cultured on CNT-coated dishes under standard culture

1 conditions were observed using an optical microscope and SEM. Saos-2 cells, a human
2 osteosarcoma cell line, were used as osteoblast-like cells in this study [27]. Fig. 4 shows the
3 optical microscope and SEM images demonstrating the morphology of Saos-2 cells cultured
4 on CNT-coated dishes after 5 days. The left column in Fig. 4 shows the optical microscope
5 images of the cells. Because the thin SWNT0.5 and MWNT0.5 films have a high
6 transparency, the morphology of the cells on these coated dishes can be recognized even
7 without staining the cells during cell culturing. Compared to the thin films, the optical
8 microscope images of the thick SWNT5 and MWNT5 films were dark, but the morphology
9 of the cells stained by Giemsa staining could be observed. There were no large aggregated
10 CNTs observed as a black area of more than approximately $1 \mu\text{m}^2$. The morphology of the
11 cells on the thin SWNT0.5 and MWNT0.5 films was not markedly different from that of the
12 cells on Culture PS. Most of the cells on the thin films were flat and well spread in all
13 directions; however, the cells on the thick SWNT5 and MWNT5 films did not appear to
14 spread more effectively than the cells on other substrates and tended to be elongated in one
15 direction. Elongation of the cells was observed more on MWNT5 than on SWNT5. The
16 middle column in Fig. 4 shows the SEM images of Saos-2 cells on CNT-coated dishes at a
17 lower magnification. The Saos-2 cells on the thin CNT films were well spread with numerous
18 cytoplasmic extensions and were flat. In contrast, the cells cultured on the rough surfaces of
19 the thick MWNT5 films exhibited a relatively elongated shape in one direction with long,

1 rounded cytoplasmic extensions. The right column in Fig. 4 shows the SEM images of the
2 peripheral part of a cell on the CNT-coated dish at a higher magnification. In the enlarged
3 image, numerous filopodia were extended onto the CNT network. The diameter of the
4 filopodia was comparable to that of the CNTs, and the apex of a filopodium was attached to
5 the surface of the CNTs. Comparison of the CNT types reveal that the cells exhibited similar
6 morphology on the different CNT types. The SEM images of the cell periphery on thick
7 MWNT films revealed that several filopodia extended from the cells toward the inside
8 reticular three-dimensional network of the thick films. This feature of thick MWNT films
9 appeared to function as a grip for high cell adhesion.

10

11 *3.3. Effect of FBS concentration on proliferation*

12

13 After the initial evaluation of the functions of CNT-coated dishes under standard culture
14 conditions, we studied the effect of the concentration of FBS on cell proliferation on
15 CNT-coated dishes. In addition to the effect on cell morphology, CNT coating may affect
16 different aspects of cell behaviors, such as adhesion and proliferation. The nanostructure of
17 CNT coating, with its large surface area and high protein adsorption activity, may affect cell
18 proliferation in different concentrations of FBS. To evaluate cell proliferation, Saos-2 cells
19 were seeded onto 60-mm tissue culture dishes at a low density of 1000 cells/cm². The

1 functions of cells at a low density are known to be influenced more by environmental
2 conditions, such as substrates and culture media, than by intercellular communication [25]. In
3 Fig. 5a, the results of cell proliferation assays on CNT-coated dishes in 1–20% FBS are
4 compared with cells grown on commercially available culture dishes, including Normal PS,
5 Culture PS, Collagen PS, and Poly-Lys PS, which are well known for cell culture. There were
6 significant differences in the proliferation rates of Saos-2 cells cultured on different dishes
7 and in different concentrations of FBS. The CNT coating on Normal PS dishes, which has a
8 low adhesiveness for cells, provided a positive effect on Saos-2 cell proliferation as compared
9 to Normal PS alone, at all FBS concentrations ($p < 0.05$). In the highest FBS concentration
10 (20%), the numbers of cells on CNTs was significantly higher ($p < 0.05$) than those on other
11 substrates, including Normal PS, Culture PS, Collagen PS, and Poly-Lys PS. Both CNT types
12 showed higher cell proliferation, which was at a similar level. No significant difference ($p >$
13 0.05) was observed in cell proliferation for either the coating amount of CNT or the type of
14 CNT. In the standard FBS concentration (10%), the number of cells on SWNTs increased
15 1.2–1.5 times more than that on Culture PS. The number of cells on MWNTs was similar to
16 that on Culture PS. On comparing the thin and thick films of each CNT types, it seems likely
17 that the increase in the number of cells on the thick films was slightly higher than that on the
18 thin films, but it was not significantly different ($p > 0.05$). Interestingly, in the lowest FBS
19 concentration (1%), the number of cells on SWNTs markedly increased as compared to that

1 on other substrates ($p < 0.05$). The highest number of cells was found on SWNT0.5. The
2 number of cells on SWNT0.5 was approximately 2.5–3.5 times higher than those on
3 MWNT0.5 and Culture PS (Figs. 5a and 5b). In addition, thin SWNT films promoted cell
4 proliferation slightly more than thick films, but no significant difference was found ($p > 0.05$).
5 In contrast, the cells grown on other substrates did not show good proliferation, and the cell
6 number did not largely exceed the number of seeded cells in 1% FBS. In the absence of FBS
7 (0%), all of the cells on all types of dishes died within approximately 6 days without
8 proliferating (data not shown). Fig. 5c shows the normalized proliferation ratio of Saos-2
9 cells grown in 1–20% FBS on the thin SWNT0.5 and MWNT0.5 films versus Culture PS.
10 The proliferation ratio was normalized with respect to the cell numbers as a control of cells
11 observed on Culture PS. The proliferation ratio for SWNT0.5 was markedly increased at low
12 FBS concentrations, while the ratio for MWNT0.5 was similar to that of Culture PS.
13 Interestingly, the changes in the ratio were not linear against the changes in serum
14 concentration.

15

16 *3.4. Protein adsorption on the surface of CNT-coated dishes*

17

18 CNTs have the ability to adsorb various molecules nonspecifically. The surface of CNTs was
19 reported to be coated with various adsorbed molecules [14,28–30]. It is very likely that the

1 proteins adsorbed from the serum onto the CNTs influence cell proliferation [31]; therefore,
2 we evaluated protein adsorption onto the surface of CNT-coated dishes. To evaluate protein
3 adsorption, we carried out adsorption assay of fluorescent-labeled protein. Fig. 6 compares
4 the fluorescence intensity of the FITC-labeled BSA solution in the dishes against different
5 incubation times. Fluorescence intensity reduced in all types of dishes with increase in
6 incubation time. Marked differences among the CNT-coated dishes, except for MWNT5,
7 were not observed. The fluorescence intensity of the labeled BSA solution in CNT-coated
8 dishes was reduced greater than that in Culture PS dishes, while the fluorescence intensity of
9 the labeled BSA solution in CNT-coated dishes showed a reduction similar to that observed in
10 Normal PS dishes. The fluorescence intensity in MWNT5, in particular, was reduced greater
11 than that in other dishes. A decrease in fluorescence intensity implies an increase in the
12 amount of adsorption of FITC-BSA onto the surface of the dishes. Thus, the surface of all
13 types of CNT-coated dishes had strong protein adsorption abilities similar to that of Normal
14 PS. Thick MWNT5 films, in particular, had a greater adsorption capacity than the other
15 CNT-coated dishes.

16

17 *3.5. Wettability of the surface before and after immersion in FBS*

18

19 Surface wettability is affected not only by surface chemistry but also by topographical

1 parameters such as roughness and texture. Surface wettability may affect the proliferation of
2 cells because the initial phase of attachment involves the physicochemical linkages between
3 cells and surfaces through ionic forces or indirectly through an alteration in the adsorption of
4 conditioning molecules, e.g., proteins. To evaluate surface wettability of CNT-coated dishes
5 before and after immersion in FBS, contact angles of dropped water on the surface were
6 measured.

7 Fig. 7 shows the contact angles of the surface of CNT-coated dishes before and after
8 immersion in 1% FBS. Before immersion, the contact angles of all CNTs were in the range of
9 84° – 108° . In contrast, those of Normal PS and Culture PS dishes were 64° and 60° ,
10 respectively. Because a higher contact angle indicates higher hydrophobicity, all CNT-coated
11 dishes exhibited more hydrophobicity than Normal PS and Culture PS dishes. Thus, the
12 surfaces of CNT-coated dishes were hydrophobic despite the different types of CNTs and
13 surface roughness before immersion in FBS. The contact angle showed a tendency to increase
14 with higher surface roughness of the CNT surface for all CNT types. No statistically
15 significant difference was observed among the SWNT groups ($p > 0.05$). After immersion,
16 the contact angles of all CNTs significantly decreased in the range of 40° to 47° (a decrease
17 of 37° – 61°) as compared to the contact angles before immersion in FBS. Thus, CNT-coated
18 dishes after immersion exhibited hydrophilicity. In contrast, the changes in contact angles on
19 Normal PS and Culture PS dishes were small (a decrease of 12°), i.e., their surfaces also

1 exhibited hydrophilicity. The contact angles of the other dishes, except SWNT0.5, showed a
2 slight difference in a narrow range from 47° to 50°. No statistically significant difference was
3 observed among the different groups, except for SWNT0.5 and Normal PS. SWNT0.5
4 exhibited the lowest contact angle of 40° in all types of dishes ($p < 0.05$), as shown in Figs.
5 7a and 7b.

6 These results suggest that the wettability of CNTs was largely affected before and after
7 immersion in FBS, and the protein-adsorbed surface of CNTs exhibited hydrophilicity. In
8 contrast, the wettability of Culture PS and Normal PS was slightly affected by immersion in
9 FBS, but they also exhibited hydrophilicity.

10

11

12 **4. Discussion**

13

14 In the present study, we prepared CNT-coated dishes with homogeneous thin or thick films by
15 coating non-modified CNTs on the surface of a commercially available nontreated
16 polystyrene dish (Normal PS). The main advantages of the CNT-coated dishes were as
17 follows: (1) coating with non-modified CNTs and without binder, (2) coating using a low
18 toxic solvent, (3) fewer aggregated CNTs and high transparency, and (4) no influence from
19 the base substrate (Normal PS as a base material has a low adhesiveness for cells). Therefore,

1 we can evaluate the natural properties of CNT films for cell responses. Thin SWNT and
2 MWNT films are promising materials because they have few aggregated CNTs and a low
3 surface roughness, which are known to largely influence cell functions.

4 The morphology and adhesion of Saos-2 cells on CNT-coated dishes were influenced more
5 by the surface roughness than by the difference in CNT types in 10% FBS. Initially, the
6 morphology and the adhesion form of Saos-2 cells cultured on CNT-coated dishes under
7 standard culture conditions were observed in Fig. 4. Most of the cells on thin SWNT0.5 and
8 MWNT0.5 films were flat and well spread in all directions. However, the cells on thick
9 SWNT5 and MWNT5 films did not spread more effectively than the cells on other substrates
10 and tended to be elongated in one direction (Fig. 4, left column). Comparison of the CNT
11 types revealed that the cells exhibited similar morphology on the different CNT types. The
12 SEM images (Fig. 4, right column) of the cell periphery on thick MWNT films revealed that
13 several filopodia were extended from the cells toward the inside the reticular
14 three-dimensional network of the thick films. This feature of thick MWNT films seems to
15 function as a grip for high cell adhesion. In general, the effect of surface roughness on the
16 morphology of osteoblast-like cells has not yet been clearly understood. Osteoblastic cells
17 spread less on the rougher surfaces than on the smoother surfaces of titanium substrates
18 [32,33], while the surface roughness of nickel-titanium alloys do not influence the
19 morphology of osteoblasts [34]. Our observations on the morphology of elongated and

1 rounded Saos-2 cells on thick SWNT and MWNT films were in agreement with the rounded
2 morphology of osteoblastic cells cultured on MWNT films and constructs [10,35,36].
3 However, on thin SWNT and MWNT films, Saos-2 cells were flat and spread out. Thus, the
4 morphology of the Saos-2 cells on CNT-coated dishes was influenced more by surface
5 roughness than by the difference in CNT type. In all types of CNT coating, CNTs can
6 significantly affect the morphology of the cells because the cells cultured on Normal PS as
7 the base substrate were not adequately spread (data not shown). These results support the
8 view that thin CNT films play an important role in the spreading of cells as an adhesive
9 scaffold without a high influence by the surface roughness.

10 In low concentrations of FBS, the proliferation ratio of the Saos-2 cells on CNT-coated
11 dishes was influenced more by the difference in CNT types than by surface roughness. In this
12 study, we showed that the ratio of Saos-2 cell proliferation was strongly affected by the
13 concentration of serum, as seen in Fig. 5. There were significant differences in the
14 proliferation of Saos-2 cells cultured on different dishes and in different concentrations of
15 FBS. The CNT coatings promoted Saos-2 cells proliferation at all concentrations of FBS.
16 Interestingly, thin SWNT films were the most effective substrate for Saos-2 cells proliferation
17 in low concentrations of FBS. There was no significant difference in the number of cells
18 present on thick and thin CNT films at all concentrations of FBS. The changes in the ratio
19 were not linear against the changes in serum concentration, as shown in Fig. 5c. Thus, cell

1 proliferation on CNTs may be influenced by some factors such as CNT type, surface
2 roughness, and the adsorption of proteins from FBS. The degree of influence of these factors
3 would change depending on the concentration of FBS. In general, the highest proliferation
4 rate was observed at the optimum roughness. Osteoblastic cells proliferation is known to be
5 influenced by the nano/microscale surface roughness of tissue culture polystyrenes with
6 varying degrees of surface roughness [37]. The results of this previous study indicated that
7 surface roughness alone caused an increase in osteoblastic cells proliferation, with the
8 greatest number of cells found on the surface with an R_a of 0.81 μm . Because our CNT-coated
9 dishes have surface roughness in the narrow range of 7–45 nm (see Table 1), surface
10 roughness would be a minor factor for proliferation. The differences between the thin and
11 thick CNT films slightly affected cell proliferation. On the other hand, from the present
12 results, it appears that the rough surface (SWNT5 and MWNT5) in the same CNT type
13 slightly increased and decreased proliferation in 10% and 1% FBS, respectively (see Fig. 5a).
14 This observation also suggests that cell proliferation on CNTs may be influenced by some
15 other factors, the degree of which would change depending on the concentration of FBS.
16 Nonetheless, these results indicate that CNT coating promotes Saos-2 cells proliferation at all
17 concentrations of FBS and that thin SWNT films, in particular, can effectively promote the
18 proliferation of Saos-2 cells at low concentrations of FBS.

19 The strong protein adsorption ability of CNT films is one of the possible reasons for CNTs

1 being a proliferation promotion factor; CNTs also function as a strong adsorbent of proteins
2 at all FBS concentration. However, the adsorption of BSA did not directly correlate with the
3 effective promotion of cell proliferation on thin SWNT films at low concentrations of FBS.
4 Fig. 6 showed that the surfaces of all types of CNT-coated dishes have strong protein
5 adsorption ability. Marked adsorption differences among the CNT-coated dishes, except for
6 MWNT5, were not observed. Thick MWNT5 films, in particular, had a greater adsorption
7 capacity than the other CNT-coated dishes. The results for the adsorption of BSA onto
8 CNT-coated dishes are in agreement with previously reported results for the adsorption of
9 BSA onto SWNTs and MWNTs [38–41]. Generally, with regard to surface chemistry,
10 proteins get adsorbed onto hydrophobic surfaces more easily. CNTs are known to be
11 hydrophobic because of their graphite structure and small diameter with a high surface energy.
12 Therefore, these factors led to the high protein adsorption of CNT-coated dishes. In addition,
13 thick MWNT films have a high surface area because of both the small diameter of CNTs and
14 porosity of the stereoscopic network structure. Therefore, thick MWNT films exhibited the
15 highest capacity for protein adsorption in the dishes. These results suggest a possible reason
16 for CNTs being a proliferation promotion factor; CNTs also functions as a strong adsorbent of
17 proteins at all FBS concentration. However, the highest capacity of protein adsorption by
18 thick MWNT films does not directly correlate with the promotion of cell proliferation at low
19 concentration of FBS, from the viewpoint of effective promotion of cell proliferation by

1 SWNTs (see Fig. 5a). Casey *et al* reported that the components of the cell culture medium
2 and the FBS growth supplement interact with SWNTs, and these interactions affect cell
3 responses [42]. Khang *et al* reported that the adsorption of cell-adhesive proteins on a CNT
4 composite was affected by nanoscale roughness and surface energy [43]. Moreover, Aoki *et*
5 *al* reported that in addition to surface topography, the adsorption of proteins from cell culture
6 medium might further enhance cell proliferation and growth [14]. A possible explanation
7 might be that the difference in cellular response to the CNTs was caused by the differences in
8 the level of protein adsorption. In this study, we expected that cell proliferation on
9 CNT-coated dishes would be strongly affected by the proteins present in the serum. Although
10 protein adsorption is considered as one of the possible factors that influence the promotion of
11 cell proliferation on CNT-coated dishes, other factors such as selectivity of adsorption,
12 conformational change in the adsorbed protein, and adsorption kinetics of proteins from
13 serum are also expected to influence the promotion of cell proliferation.

14 The wettability of CNT films before immersion in FBS is a possible reason for CNTs being
15 a proliferation promotion factor at 10% and 20% FBS. However, at present it is unclear if the
16 lowest wettability of the thin SWNT films after immersion in FBS directly correlates with
17 effective promotion of cell proliferation on thin SWNT films at low concentrations of FBS.
18 We showed that the wettability of CNTs was largely affected before and after FBS immersion.
19 The surfaces of the CNT-coated dishes were hydrophobic despite different CNT types and

1 surface roughness before immersion in FBS. The wettability of the surfaces before immersion
2 seems to be relative to the ratio of cell proliferation in 10% and 20% FBS (Fig. 5a, top and
3 middle); however, it was not directly relative to the ratio of cell proliferation in 1% FBS (Fig.
4 5a, bottom). After immersion in FBS, the surfaces of the CNT-coated dishes exhibited
5 hydrophilicity. The surface of all types of CNT-coated dishes had strong protein adsorption
6 ability, as shown in Fig. 6. Therefore, the protein-adsorbed surface of CNTs exhibited
7 hydrophilicity. Van Wachem *et al* reported that moderately wettable surfaces show good
8 adhesion of cells. Both greater hydrophilic and greater hydrophobic surfaces show less
9 adhesion [44]. In this study, CNT-coated dishes exhibited good Saos-2 cells proliferation
10 because of their moderate wettability after immersion in FBS. Although there were slight
11 differences in the wettability of all types of dishes, SWNT0.5 exhibited the lowest contact
12 angle of 40°, as shown in Fig. 7a and 7b. One possible reason for the high levels of cell
13 proliferation observed on the thin SWNT films may be that these films have moderate
14 wettability that allows cell proliferation in low concentrations of serum because of both
15 adequate surface roughness at the nanoscale and strong protein adsorption. Nonetheless,
16 wettability cannot explain the coherent mechanism of the effective promotion of cell
17 proliferation by thin SWNT films in low concentrations of FBS because of the slight
18 differences observed.

19 In this study, we showed that the ratio of proliferation of Saos-2 cells on CNT-coated

1 dishes was strongly affected by the concentration of serum. Interestingly, thin SWNT films
2 were the most effective substrates for the proliferation of Saos-2 cells in low concentrations
3 of FBS. Thus far, little is known about the behavior of cells in media with low serum
4 concentrations [18–20,44–47], in particular, the relationship of cell proliferation with various
5 substrates. To our knowledge, the effect of CNT coating on cell proliferation in low serum
6 concentrations has not been studied. At present, we cannot explain the coherent mechanism
7 of the excellent promotion of cell proliferation by thin SWNT films in low concentrations of
8 FBS from the results of (a) surface roughness and topography, (b) protein adsorption of BSA,
9 and (c) wettability before and after immersion in FBS.

10 Although the mechanism of the promotion of cell proliferation on CNT-coated dishes is
11 still poorly understood, we propose a possible mechanism of the promotion of proliferation of
12 Saos-2 cells on CNT-coated dishes. Initially, the surface of the CNT-coated dishes has
13 adsorbed proteins, and the resulting CNT surface has a moderate wettability. Thus, the
14 adsorbed proteins play an important role in the proliferation of Saos-2 cells. Previous reports
15 also indicate that protein adsorption plays a crucial role in cell proliferation on CNT
16 substrates [14,31]. Both SWNTs and MWNTs function as adsorbents under standard cell
17 culture conditions and low concentrations of FBS. In particular, the moderate roughness at
18 the nanoscale level and moderate wettability after immersion in FBS of thin SWNT films
19 may promote the proliferation of Saos-2 cells without being affected by the rough surface of

1 the three-dimensional network of MWNTs.

2 Another possible reason for the high level of cell proliferation observed on thin SWNT
3 films may be the selective adsorption of cell-adhesive proteins such as fibronectin,
4 vitronectin, laminin, and so on from the cell culture medium. SWNT films may selectively
5 adsorb the specific protein(s) necessary for the proliferation of Saos-2 cells from the cell
6 culture medium, and the function of the adsorbed protein on SWNT film is enhanced at lower
7 concentrations of FBS. Then, the rough surface of the thick SWNT films can slightly disturb
8 the cell proliferation in low concentrations of FBS. Thus, thin SWNT films may be the most
9 effective substrate for the proliferation of Saos-2 cells in low concentrations of FBS. Grinnell
10 *et al* [45] reported that fibronectin adsorption was markedly decreased for serum
11 concentrations above 1% compared to serum concentrations below 1% and that fibronectin
12 adsorption occurred more on hydrophobic surfaces than on hydrophilic surfaces. Their
13 finding supports the view that the high hydrophobicity of CNTs promotes cell proliferation in
14 low concentrations of FBS. Khang *et al* reported that the adsorption of cell-adhesive proteins
15 on a CNT composite surface was affected by its nanoscale roughness and surface energy [43].
16 From their findings, it can be said that the nanoscale roughness and surface energy of thin
17 SWNT films may play a role in the selective adsorption of cell-adhesive proteins. Jia *et al*
18 reported that the function of insulin-like growth factor on osteoblastic differentiation was
19 generally greater in media containing lower serum concentrations [48]. Gowen *et al* reported

1 that the function of interleukin-1 on osteoblastic proliferation was enhanced in low serum
2 concentrations [49]. These findings indicate that the function of the adsorbed protein on
3 SWNT films may be enhanced in lower concentrations of FBS.

4 Further work is underway to clarify the selective adsorption of cell-adhesive proteins and
5 the role of the adsorbed proteins on CNT-coated dishes. Our new findings on the functions of
6 CNTs indicate that thin SWNT films may be used as an effective culture substrate for
7 culturing Saos-2 cells in low concentrations of serum. In future, we should investigate this
8 effect with several types of cells.

9

10

11 **5. Conclusion**

12

13 In this study, we prepared culture dishes with homogeneous thin or thick films of
14 nonmodified CNTs and examined the effects of serum concentrations on human osteoblastic
15 cells (Saos-2) proliferation in these culture dishes. The results of cell proliferation assays on
16 CNT-coated dishes in 1–20% FBS were compared with those assays on commercially
17 available culture dishes, including Normal PS, Culture PS, Collagen PS, and Poly-Lys PS. In
18 this study, we showed that the ratio of cell proliferation was strongly affected by the
19 concentration of serum. Interestingly, thin SWNT films were the most effective substrate for

1 the proliferation of Saos-2 cells in low concentrations of serum. Thus, thin SWNT films may
2 be used as an effective biomaterial for Saos-2 culturing in low concentration.

3

4 **Acknowledgments**

5

6 The authors would like to thank Dr. Jin Shigeki from the Creative Research Initiative
7 “SOUSEI” at Hokkaido University for acquiring AFM images in data processing. This work
8 was partly funded by “Collaborative Development of Innovative Seeds, Potentiality
9 Verification Stage” of the Japan Science and Technology Agency (JST) and by Health and
10 Labour Science Research Grants in 2006 (H18-kagaku-006) from the Ministry of Health,
11 Labour and Welfare of Japan.

12

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3

1

2 **Table 1**

3

	Culture PS	SWNT0.5	SWNT5	MWNT0.5	MWNT5
R_a (nm)	2.2 ± 0	7.1 ± 0.6	15.8 ± 0.9	7.9 ± 1.6	45.0 ± 1.7

4 Results are presented as mean \pm standard error of 3 experiments.

5

6

7 **Table Caption**

8

9 Table 1. Roughness average (R_a) observed by AFM cross-sectional analysis of the surface of

10 CNT-coated dishes.

11

1 **Figure Captions**

2

3 Fig. 1. Photographs of CNT-coated dishes.

4 CNT-coated dishes were prepared by coating non-modified CNTs on the surface of a
5 commercially available non-treated polystyrene dish (Normal PS). Thin film of $0.5 \mu\text{g}/\text{cm}^2$
6 SWNTs (SWNT0.5), thick film of $5 \mu\text{g}/\text{cm}^2$ SWNTs (SWNT5), thin film of $0.5 \mu\text{g}/\text{cm}^2$
7 MWNTs (MWNT5), and thick film of $5 \mu\text{g}/\text{cm}^2$ MWNTs (MWNT5).

8

9 Fig. 2. AFM characterization.

10 (a) Roughness average (R_a) observed by AFM cross-sectional analysis of the surface of
11 CNT-coated dishes and summarized in Table 1. Results are presented as mean \pm SE of 3
12 experiments. AFM images ($2 \mu\text{m} \times 2 \mu\text{m}$) and cross-sectional analysis of (b) $5 \mu\text{g}/\text{cm}^2$ SWNT
13 thick films (SWNT5) and (c) $5 \mu\text{g}/\text{cm}^2$ MWNT thick films (MWNT5).

14

15 Fig. 3. SEM images of CNT-coated dishes.

16 (a) Thin film of $0.5 \mu\text{g}/\text{cm}^2$ SWNTs (SWNT0.5), (b) thick film of $5 \mu\text{g}/\text{cm}^2$ SWNTs
17 (SWNT5), (c) thin film of $0.5 \mu\text{g}/\text{cm}^2$ MWNTs (MWNT5), and (d) thick film of $5 \mu\text{g}/\text{cm}^2$
18 MWNTs (MWNT5). Scale bars indicate $1 \mu\text{m}$.

19

1 Fig. 4. Optical microscope and SEM images of Saos-2 cells on CNT-coated dishes.

2 (Left column) optical microscope images ($\times 20$) observed with the same light intensity and
3 at the same exposure time of cells stained by Giemsa staining, (middle column) SEM images
4 at 45° at a lower magnification ($\times 1000$), and (right column) SEM images at 45° at a higher
5 magnification ($\times 15000$). Saos-2 cells were cultured in DMEM containing 10% FBS for 5
6 days. Scale bars of the left, middle, and right columns are 100, 30, and 2 μm , respectively.

7

8 Fig. 5. Effects of 1–20% serum concentration on cell proliferation of Saos-2 cells cultured on
9 CNT-coated dishes.

10 (a) Proliferation of Saos-2 cells cultured on CNT-coated dishes in different concentrations
11 of FBS, namely, 20% (top), 10% (middle), and 1% (bottom), in comparison to commercially
12 available culture dishes. Error bars indicate a standard error for $n = 4$. Initial seeding density
13 was 1000 cells/cm². Dotted lines are the number of seeded cells. (b) Comparison of images of
14 Saos-2 cells cultured on thin SWNT0.5 and MWNT0.5 films in 1% FBS for 2 weeks. The
15 cells were stained with Giemsa stain. Scale bars are 300 μm . (c) Normalized proliferation
16 ratio of cells cultured on thin SWNT0.5 and MWNT0.5 films versus cell culture polystyrene
17 (Culture PS) in 1–20% FBS. The proliferation ratio was normalized with respect to the cell
18 number in the control of polystyrene dish (Culture PS). The normalized value of cell number
19 in the Culture PS dish was set as 1.

1

2 Fig. 6. Protein adsorption onto the surface of CNT-coated dishes with incubation time
3 determined by an adsorption assay of fluorescent-labeled proteins.

4 Four milliliters of a low concentration of FITC-BSA solution (1 $\mu\text{g/ml}$) were incubated in
5 60-mm CNT-coated dishes, including SWNT0.5, SWNT5, MWNT0.5, and MWNT5. After
6 incubation at room temperature, the supernatant was measured by using a fluorescence
7 microscope (Ex = 495 nm, Em = 520 nm). Cell culture polystyrene dish (Culture PS) and
8 suspension culture polystyrene dish (Normal PS) were used as controls.

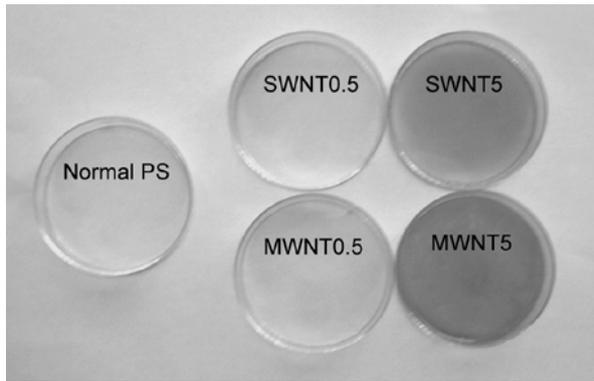
9

10 Fig. 7. Contact angles of CNT-coated dishes before and after immersion in 1% FBS.

11 (a) Contact angles on various substrates before and after immersion in 1% FBS. Error bars
12 indicate a standard error for $n = 3$. “*” indicates a significant difference at $p < 0.05$ for the
13 comparison between SWNT0.5 and the values of the other dishes after immersion. (b) Images
14 of the spreading of a water drop on a thin SWNT0.5 film after 10 s.

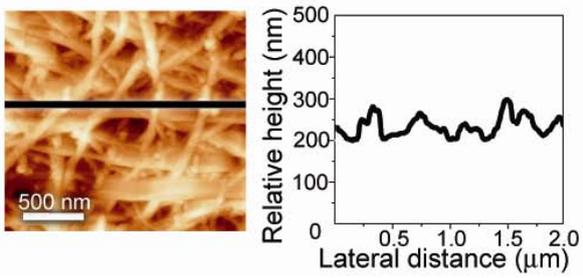
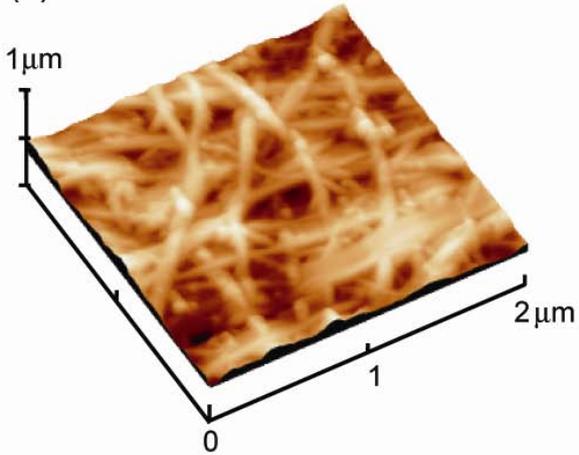
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1 **Fig. 1.**

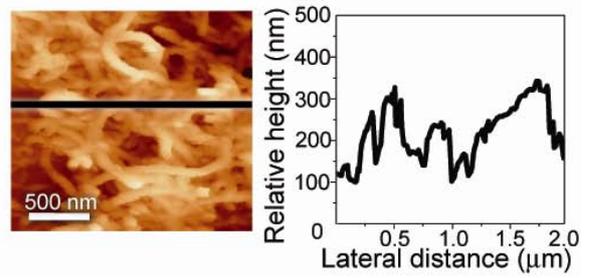
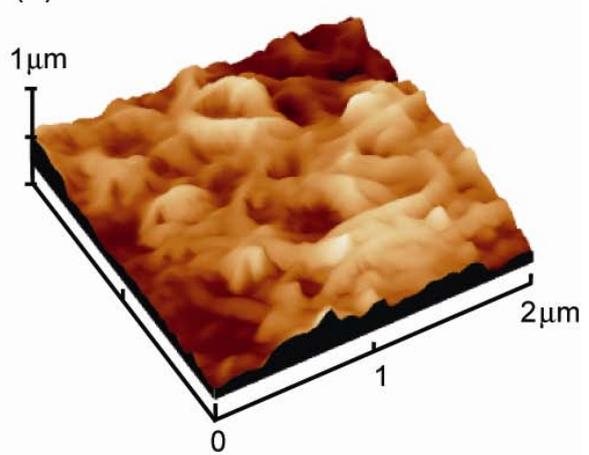


5 **Fig. 2.**

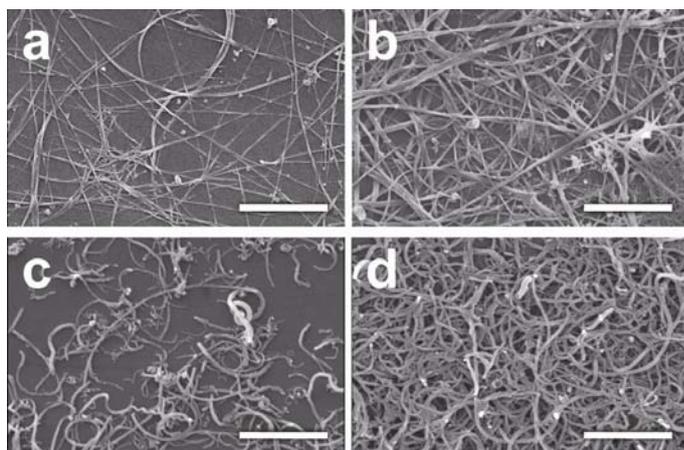
(a) SWNT5



(b) MWNT5



1 **Fig. 3.**

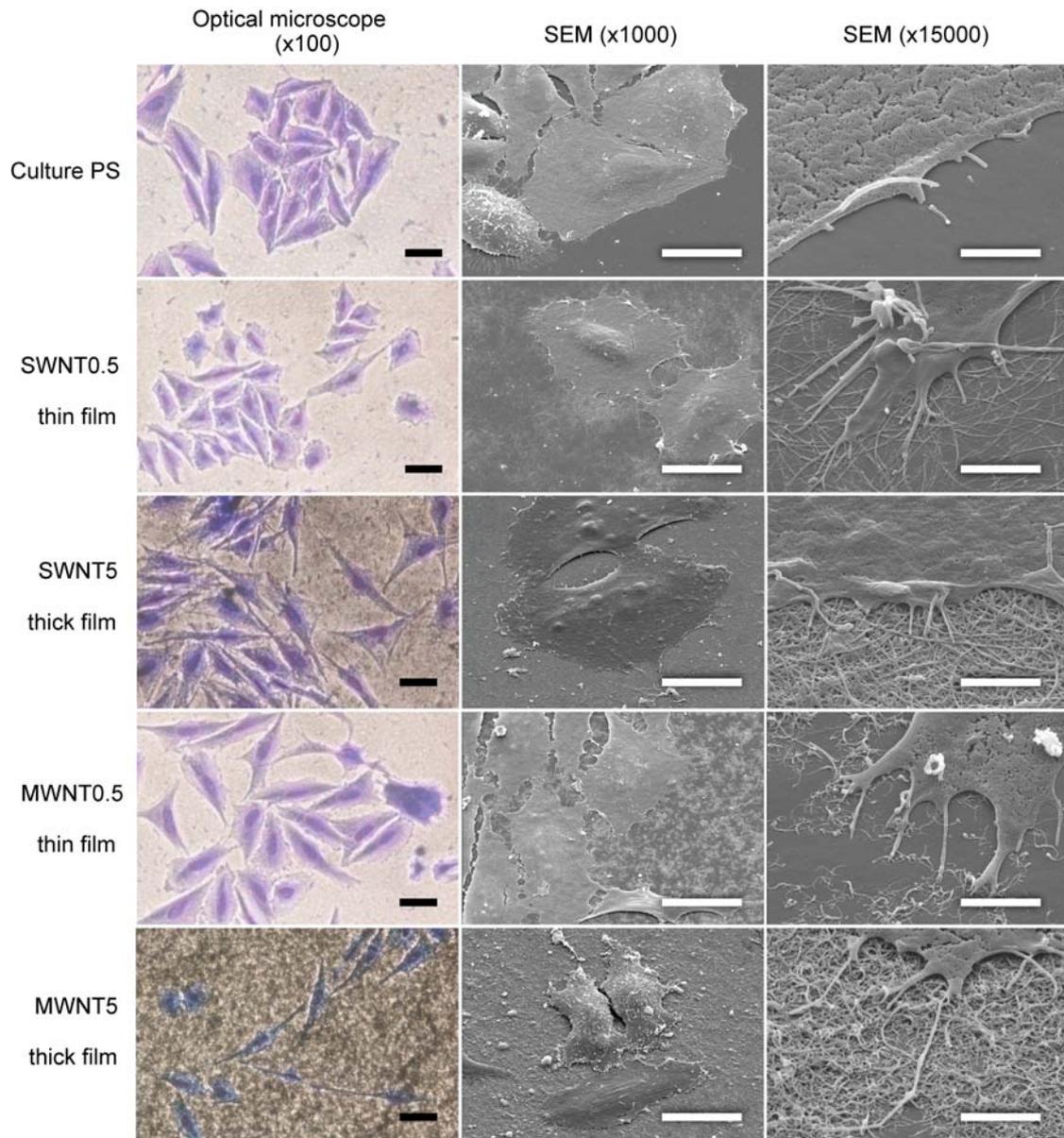


2

3

4

1 **Fig. 4.**

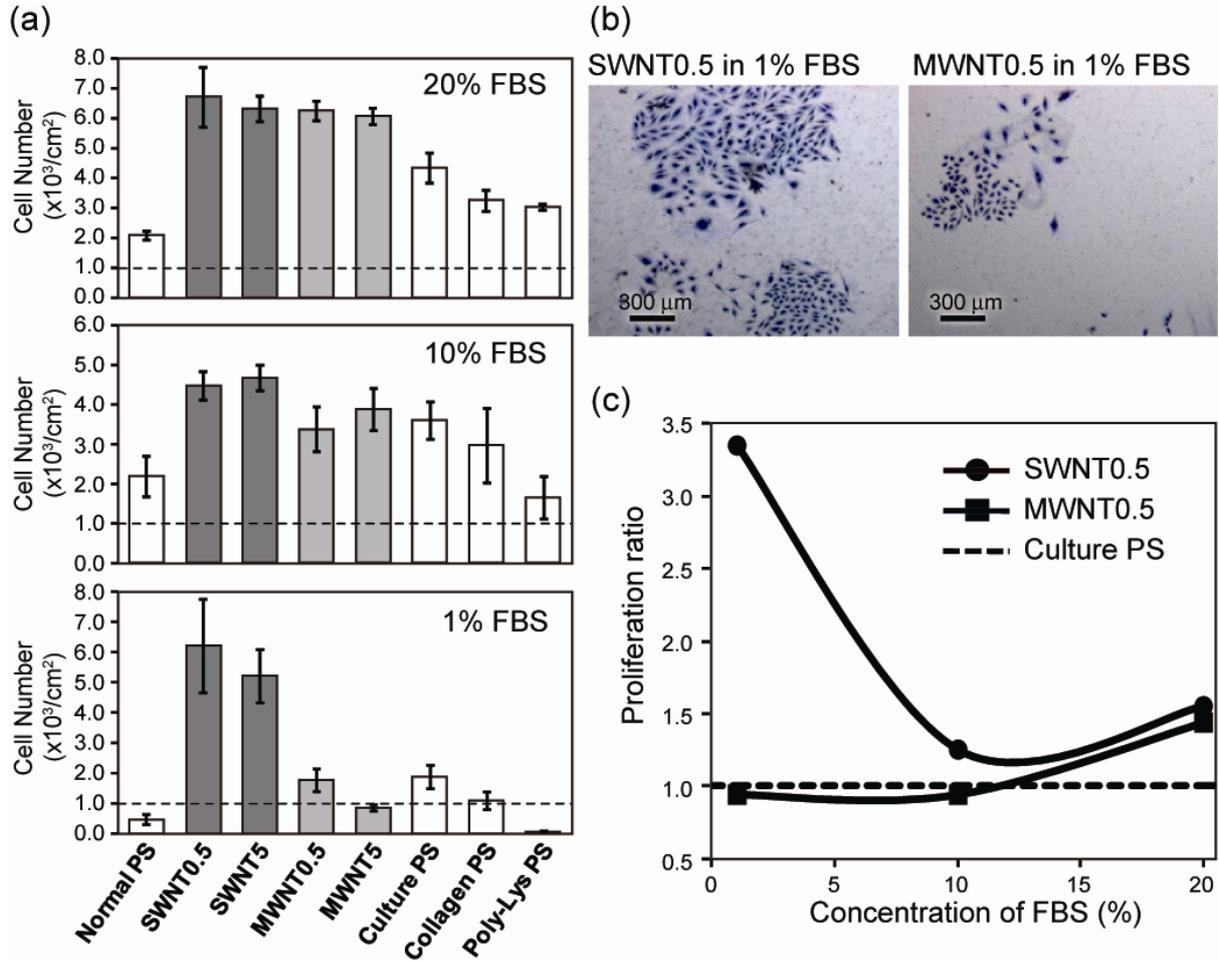


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3

4

1 **Fig. 5.**

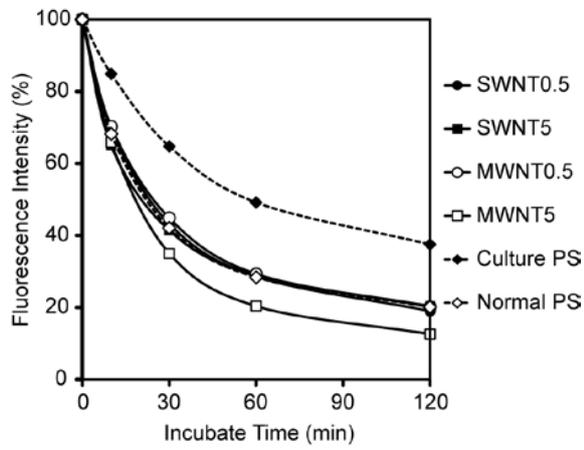


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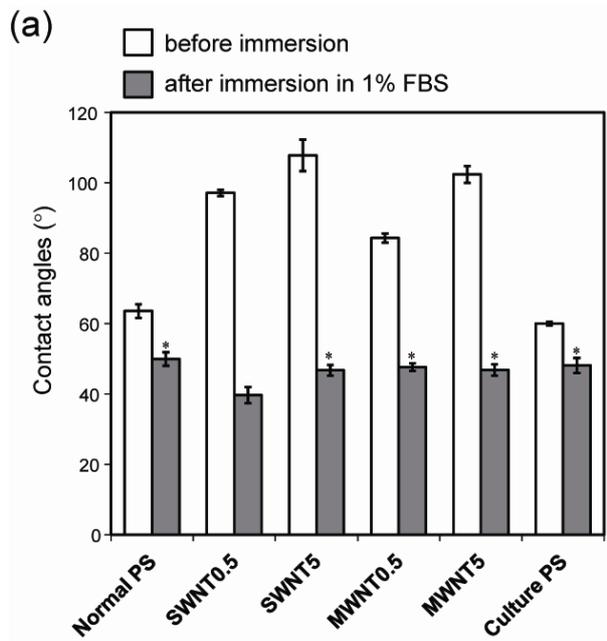
1 **Fig. 6.**



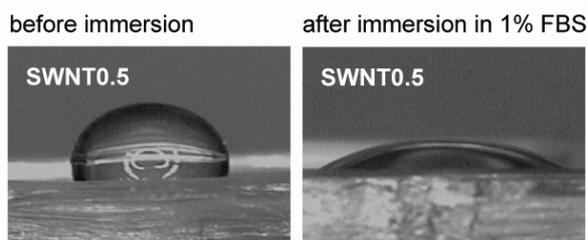
2

3

4 **Fig. 7.**



(b)



5