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Development of a Multiwalled Carbon Nanotube Coated Collagen Dish

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SYNOPSIS

Carbon nanotubes (CNTs) are one of the most interesting nanomaterials because of their excellent characteristics. In this study, a transparent CNTs coating for cell culture dishes was developed and its properties for cell culture were estimated. Carboxylated multiwalled carbon nanotubes (MWCNTs) were dispersed in aqueous sodium cholate solution and applied on a collagen type I-coated cell culture dish (cover glass). The dish surface was homogeneously covered by MWCNTs without aggregation. The MWCNT-coated dish was slightly gray and had good transparency, so conventional optical microscopic observation of the cells on the MWCNT-coated dish was possible. Rat osteoblast-like cells cultured on the MWCNT-coated dish showed slightly lower viability and proliferation compared to the collagen-coated dish. The cell adhesion on the MWCNT-coated dish was much higher than that on the collagen-coated dish. Therefore, MWCNT-coating for dishes will be a useful new material for cell culture.

INTRODUCTION

Carbon nanotubes (CNTs) and other carbon nanomaterials are of interest for biological and medical applications because of their high chemical durability, mechanical strength and electrical properties. Studies of the application of carbon nanomaterials have been carried for the substrate of cell culture¹⁻⁹⁾, drug delivery systems^{10,11)} and medical implant materials^{12,13)}.

Cells have high affinity to singlewalled CNTs (SWCNTs)^{5-7,9,14)}, multiwalled CNTs (MWCNTs)^{2,4,6-8)} and other carbon nanofibers^{1,3)}. The bone cell affinity to CNTs^{1,14)} and bone formation (osseointegration) on sintered MWCNTs were reported¹²⁾. However, those CNT-based substrates were black and had low optical transparency. Therefore, conventional optical microscopic observation of the cultured cells on the CNT-based substrates was quite difficult and the cells needed to be observed by scanning electron microscopy (SEM) after fixation. However, *in situ* microscopic observation of cultured cells on CNTs is important to evaluate the cell affinity to them.

MacDonald et al. reported a collagen-SWCNT composite for the cell culture substrate⁵⁾. The SWCNTs were strongly entrapped by collagen and the composite showed high mechanical strength and good cell viability. Then good affinity between collagen and CNTs was expected.

On the other hand, collagen is one of the most biocompatible materials, and then collagen-coated dish is widely used for cell culture. From the Macdonald's result, CNTs would show high affinity to the collagen-coated dish surface. Then above CNTs remarkable properties would be added to the collagen-coated dish. Concerning to the CNTs, purification and surface treatment was easier carried out for MWCNTs than SWCNTs because of their chemical stability resulted from thick tubular structure. Thus MWCNTs coating on the collagen-coated dish would be possible by the appropriate treatment of MWCNTs.

In this study, MWCNTs were coated onto collagen-coated cell culture, in an attempt to develop coated dishes with optically transparent MWCNTs, and the cell viability, proliferation, and adhesion on the MWCNT-coated dishes were estimated.

MATERIAL AND METHODS

Preparation of MWCNT-coated dishes and surface roughness measurement

MWCNTs (20-30nm in diameter, Cnt, Seoul, Korea) were purified by oxidation at 500°C for 90 minutes and treated in concentrated hydrochloric acid. The purified MWCNTs were carboxylated by the method reported by Peng et al.¹⁵⁾ to improve their dispersion in aqueous solutions. The carboxylated MWCNTs were dispersed in sodium cholate (1w/v%) aqueous solution to MWCNT concentrations of 1-1,000ppm under sonication for 90 minutes. Sodium cholate was reported to be one of the most effective surfactants for carbon nanotube dispersion¹⁶⁾. The obtained MWCNT suspension (2ml/dish) was poured into a collagen type I-coated cell culture dish (35mm ϕ , Iwaki, Tokyo, Japan) and kept at room temperature for 3 hours. Then it was rinsed with deionized water, dried and employed for the following cell culture experiments. For the SEM observations, a collagen type I coated cover glass (25mm ϕ , Iwaki, Tokyo, Japan) with the same treatment was used instead of the culture dish. Hereafter, the dishes and the cover glasses treated with the MWCNT suspension are referred to as “MWCNT-coated dish” and “MWCNT-coated cover glass”, respectively.

To estimate the optimum treatment conditions for the MWCNT solution, the collagen-coated dishes were treated with 1-1,000ppm suspensions in 1w/v% sodium cholate aqueous solution for 1-6 hours. The homogeneity of the MWCNT coating on these dishes was estimated by SEM observation (S-4000, Hitachi, Japan) and optimum treatment conditions were determined. The changes in surface roughness of the collagen-coated cover glass before and after MWCNT coating were estimated using a surface roughness meter (Surfcom 130A, Tokyo Seimitsu, Tokyo, Japan).

Cell proliferation, viability and adhesion on MWCNT-coated dishes

Rat osteoblast-like MC3T3-E1 cells were seeded onto MWCNT-coated dishes and collagen-coated dishes at 1×10^5 cells/2ml/dish. These cells were cultured in α -MEM (Gibco, USA) with 10% FBS (Biowest, USA) and PSN Antibiotic Mixture (Gibco, USA) at 37°C in humidified 5% CO₂ for 24, 48 and 72 hours and used for the following proliferation and viability estimations. Observation and cell counting were done under an optical microscope after fixation and Giemsa staining (Merck, USA). The number of cells per dish was estimated under optical microscopic observation and the cell proliferation was estimated. Cell viability was measured by colorimetry using Alamer blue (Biosource, USA). The cells were cultured in medium containing 10% Alamer blue and the changes in absorbance at 570nm and 600nm were estimated using a spectrophotometer (U-1100, Hitachi, Japan). Cell adhesion was

estimated by treatment using diluted Trypsin-EDTA solution (Gibco, USA). The MC3T3-E1 cells that were cultured to confluence on MWCNT-coated dishes and collagen-coated dishes were treated with 0.1% and 0.02% Trypsin-EDTA solution. The decrease of the attached cells with treatment time was evaluated under an optical microscope.

RESULTS

Fig. 1 shows SEM images of MWCNT-coated cover glasses treated with various concentrations of carboxylated MWCNT suspension. The coverage of MWCNTs on the surface was increased with the increase of the MWCNT concentration from 10ppm to 1,000ppm. In contrast, MWCNT aggregation was observed on the dish treated with 1,000ppm MWCNT suspension. Therefore, the optimum concentration of the MWCNT suspension for the coating treatment was estimated to be 100ppm. The effect of the treatment period using 100ppm suspension was also estimated and optimum MWCNT coverage was obtained after 3-hour treatment.

No significant difference was observed in the arithmetic mean surface roughness (R_a) of the collagen- and MWCNT-coated cover glass because of the detection limit. The maximum roughness (R_{max}) of the MWCNT-coated cover glass was estimated to be $0.78 \pm 0.08\mu\text{m}$ and it was slightly higher than that of the collagen-coated cover glass ($R_{max}=0.52 \pm 0.05\mu\text{m}$). Thus the MWCNT-coated surface was estimated to be as smooth as the collagen-coated surface.

The MWCNT-coated dishes used in the following experiments were treated with 100ppm MWCNT suspension for 3 hours. The MWCNTs were strongly entrapped on the collagen-coated surface and never released by rinsing or cell culture procedures.

Fig. 2 shows a comparison of the color and the transparency of the collagen-coated dish and the MWCNT-coated dish. The MWCNT-coated dish looked slightly gray but had good transparency.

Fig. 3 shows the cell proliferation on an MWCNT-coated dish and collagen-coated dish. The cells on both dishes showed similar tendencies and their difference was not significant until 48 hours (t-test, $p < 0.05$). Fig. 4 shows cell viability on both dishes. The cell viability on the MWCNT-coated dish was slightly lower than that on the collagen-coated dish.

Fig. 5 shows optical microscope images (a) and SEM images (b) of the cultured cells on MWCNT-coated and collagen-coated dishes. The changes in cell morphology on both dishes were similar in the optical images; however, the cells on the MWCNT-coated dish were not widespread as those on the collagen-coated dish. Fig. 6 shows high magnification SEM images of the filopodia of E1 cells. Large numbers of filopodia were observed in the cells on the MWCNT-coated dish and the ends of the filopodia appeared to contact MWCNTs.

Fig. 7 shows the residual cell percentage of the dish surface in the Trypsin-EDTA treatment period (values represent mean detachment of cells \pm SD from $n=4$). Cells on the MWCNT-coated dish were detached from the dish surface within a few minutes with 0.1% Trypsin-EDTA solution. Even with the lower concentration of Trypsin-EDTA (0.02%), all cells on the collagen-coated dish were detached within 10 minutes. In contrast, some of the cells on the MWCNT-coated dish remained on the surfaces of MWCNTs for more than 30 minutes of 0.1% Trypsin-EDTA treatment. Fig. 8 shows that cells were detached with 0.02% Trypsin-EDTA solution at 2 minutes. Fig. 9 shows SEM images of the cells on an MWCNT-coated dish after 2 minutes of treatment with 0.02% Trypsin-EDTA solution. Mechanical contact between the filopodia and MWCNTs could be observed.

DISCUSSION

Usually, CNTs and other carbon nanoparticles have low dispersion in aqueous solutions because of their hydrophobicity. However purification and surface treated methods were well studied for MWCNTs than SWCNTs, and carboxylated MWCNTs¹⁵⁾ can be stably dispersed into sodium cholate aqueous solution¹⁶⁾. The collagen-coated dishes could be homogeneously covered by MWCNTs using the above suspension, as shown in Fig. 1, under optimum treatment conditions (100ppm for 3 hours). SEM and surface roughness observations revealed that the collagen-coated surface was homogeneously and fully covered with MWCNTs without their aggregation. The carboxylation of MWCNTs and sodium cholate addition were effective to obtain homogeneous coverage of MWCNTs on the collagen-coated dish surface.

Concerning to the interaction between CNTs and collagen, MacDonald et al.⁵⁾ reported SWCNT-reinforced collagen. As the mechanism of SWCNT entrapment in collagen, they suggested the blend and interaction between CNTs and collagen fibrils. In the present study, MWCNTs were homogeneously coated on collagen-coated cell culture dishes and cover glass. The coated MWCNTs were strongly fixed on the collagen-coated surface. Thus, strong entrapment of MWCNTs by collagen would also have occurred via a similar mechanism

The prepared MWCNT-coated dishes showed good transparency and conventional optical microscopic observation could be easily carried out. Usually, cell culture studies on CNTs were carried out on the CNTs membranes which have no optical transparency. Therefore the observation of cells on CNT membranes should be carried out by SEM. Our MWCNT-coated dishes had a densely packed MWCNT surface with optical transparency, and optical microscopic observation of cells cultured on MWCNTs became possible. Thus, they would be suitable MWCNT substrate for cell culture study on MWCNTs.

The proliferation and viability of the cells on the MWCNT-coated dish were slightly lower than those on the collagen-coated dish as shown in Fig. 3 and Fig. 4. In addition, the cells on the MWCNT-coated dish were widely extended on that dish surface (Fig. 5). The cell adhesion on the MWCNT-coated dish was quite strong as shown in Fig. 7. Aoki et al.⁸⁾ reported that the cells on an MWCNT membrane were strongly attached and were not detached by trypsin treatment. That was in good agreement with our results. As shown in Fig. 9, mechanical contact between MWCNTs and pseudopods of the cells was observed. Aoki et al.⁸⁾ and Zanello et al.¹⁴⁾ also reported the same mechanical contact of bone cells cultured on MWCNTs. This mechanical binding would be one reason for the high adhesion, and the large specific surface area of MWCNTs would also be effective to increase the adhesion.

There were many reports of the interaction between SWCNTs and MWCNTs and variety of cells. Hu et al.⁹⁾ reported good neural cell viability on MWCNTs deposited on the polyethyleneimine-coated cover glass. Aoki et al.⁸⁾ and Zanello et al.¹⁴⁾ reported the comparison of bone cell proliferation on various CNTs. Aoki et al.⁷⁾ found the highest cell proliferation and viability on the SWCNT membranes. Those for MWCNTs were lower than for SWCNTs but higher than for graphite particles. Zanello et al.¹⁴⁾ also reported higher cell proliferation on SWCNTs than on MWCNTs, but the osteoblasts cultured on MWCNTs showed an osteocyte-like shape. That suggested the differentiation of osteoblasts on MWCNTs. Thus the interaction between various cells and CNTs was strongly interested. However, most of CNTs substrates had no optical transparency, and then cell observation should be carried with SEM. This means the difficulty of the conventional cell observation *in situ* with optical microscope while cell cultured. Our aim was to prepare the CNTs based cell culture substrate which is applicable for ordinary cell culture and optical microscope observation. The obtained MWCNT-coated dish in this study had densely MWCNT-coated surface with good optical transparency. Then, cell morphology and behavior on MWCNTs while cultivation could be observed with optical microscope *in situ*. The cell proliferation and viability of the cells on those dish was comparable to those of the collagen-coated dish which is known as the one of the best substrate for cell culture. In addition, cell adhesion on the MWCNT-coated dish was extremely stronger than the collagen-coated dish. The preparation of the present MWCNT-coated cell culture materials was quite simple and it would be

applicable for other carbon nanomaterials e.g. SWCNTs or carbon nanohornes. Thus, the feasibility of the MWCNT-coated dish for the application of cell culture on the MWCNTs and other carbon nanomaterials was suggested.

The MWCNT-coated dish prepared in the present study would provide cell proliferation and viability of the cells comparable to those of the collagen-coated dish. And cell adhesion on the MWCNT-coated dish was extremely stronger than the collagen-coated dish. The collagen-coated dish is one of the best substrates for cell culture. The preparation of the present MWCNT-coated cell culture materials was quite simple and showed good transparency. Thus, the feasibility of the MWCNT-coated dish for cell culture was suggested.

CONCLUSION

Carboxylated MWCNTs were homogeneously coated on collagen-coated cell culture dishes. The MWCNTs were strongly entrapped on the collagen-coated dish surface and the dishes had good optical transparency. Thus, in situ optical microscope observation of cultured cells on the MWCNTs was possible. The viability and proliferation of MC3T3-E1 cells on MWCNT-coated dishes were comparable to those on dishes coated with collagen, which is one of the most appropriate substrates for cell culture. The MWCNT-coated dishes had high cell viability comparable to that on collagen-coated dishes, and the cell adhesion on the MWCNT-coated dishes was quite strong compared to that on the collagen-coated dishes. SEM images suggested that one of the reasons for the strong cell adhesion on MWCNT-coated dishes was the mechanical contact between MWCNTs and pseudopods. Therefore, the coating of carboxylated MWCNTs on collagen-coated dishes will be useful not only for in situ cell observation on CNTs but also for the improvement of cell adhesion.

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REFERENCES

- 1) Price RL, Waid MC, Haberstroh KM, Webster TJ. Selective bone cell adhesion on formulations containing carbon nanofibers. *Biomaterials* 2003; 24: 1877-1887.
- 2) Hu H, Ni Y, Montana V, Haddon RC, Parpura V. Chemically functionalized carbon nanotubes as substrates for neuronal growth. *Nano Lett.* 2004; 4: 507-511.
- 3) McKenzie JL, Waid MC, Shi R, Webster TJ. Decreased functions of astrocytes on carbon nanofiber materials. *Biomaterials* 2004; 25: 1309-1317.
- 4) Correa-Duarte MA, Wagner N, Rojas-Chapana J, Morszeck C, Thie M, Giersing M. Fabrication and biocompatibility of carbon nanotube-based 3D networks as scaffolds for cell seeding and growth. *Nano Lett.* 2004; 4: 2233-2236.
- 5) MacDonald RA, Laurenzi BF, Viswanathan G, Ajayan PM, Stegemann JP. Collagen-carbon nanotube composite materials as scaffolds in tissue engineering. *J. Biomed. Mater. Res., Part A* 2005; 74A: 489-496.
- 6) Hu H, Ni Y, Mandal SK, Montana V, Zhao B, Haddon RC, Parpura V. Polyethyleneimine functionalized single-walled carbon nanotubes as a substrate for neuronal growth. *J. Phys. Chem. B* 2005; 109: 4285-4289.
- 7) Aoki N, Akasaka T, Watari F, Yokoyama A. Carbon nanotubes as scaffolds for cell culture and effect on cellular functions. *Dent. Mater. J.* 2007; 26: 178-185.
- 8) Aoki N, Akasaka T, Yokoyama A, Nodasaka Y, Akasaka T, Uo M, Sato Y, Thoji K, Watari F. Cell culture on a carbon nanotube scaffold. *J. Biomed. Nanotechnol.* 2005; 1: 402-405.
- 9) Jan E, Kotov NA. Successful differentiation of mouse neural stem cells on layer-by-layer assembled single-walled carbon nanotube composite. *Nano Lett.* 2007; 7: 1123-1128.
- 10) Ajima K, Yudasaka M, Maigné A, Miyawaki J, Iijima S. Effect of functional groups at hole edges on cisplatin release from inside single-wall carbon nanohorns. *J. Phys. Chem. B* 2006; 110: 5773-5778.
- 11) Feazell RP, Nakayama-Ratchford N, Dai H, Lippard SJ. Soluble single-walled carbon nanotubes as longboat delivery systems for platinum (IV) anticancer drug design. *J. Am. Chem. Soc.* 2007; 129: 8438-8439.
- 12) Wang W, Watari F, Omori M, Liao S, Zhu Y, Yokoyama A, Uo M, Kimura H, Ohkubo A. Mechanical properties and biological behavior of carbon nanotube/polycarbosilane composites for implant materials. *J. Biomed. Res., Part B* 2007; 82B: 223-230.
- 13) Usui Y, Aoki K, Narita N, Murakami N, Nakamura I, Nakamura K, Ishigaki N, Yamazaki H, Horiuchi H, Kato H, Taruta S, Kim YA, Endo M, Saito N. Carbon nanotubes with high bone-tissue compatibility and bone-formation acceleration effects. *Small* 2008; 4: 240-246.
- 14) Zanello LP, Zhao B, Hu H, Haddon RC. Bone cell proliferation on carbon nanotubes. *Nano Lett.* 2006; 6: 562-567.
- 15) Peng H, Alemany LB, Margrave JL, Khabashesku VN. Sidewall carboxylic acid functionalization of single-walled carbon nanotubes. *J. Am. Chem. Soc.* 2003; 125: 15174-15182.
- 16) Ishibashi A, Nakashima N. Individual dissolution of single-walled carbon nanotubes in aqueous solutions of steroid or sugar compounds and their raman and near-ir spectral properties. *Chem. --Eur. J.* 2006; 12: 7595-7602.

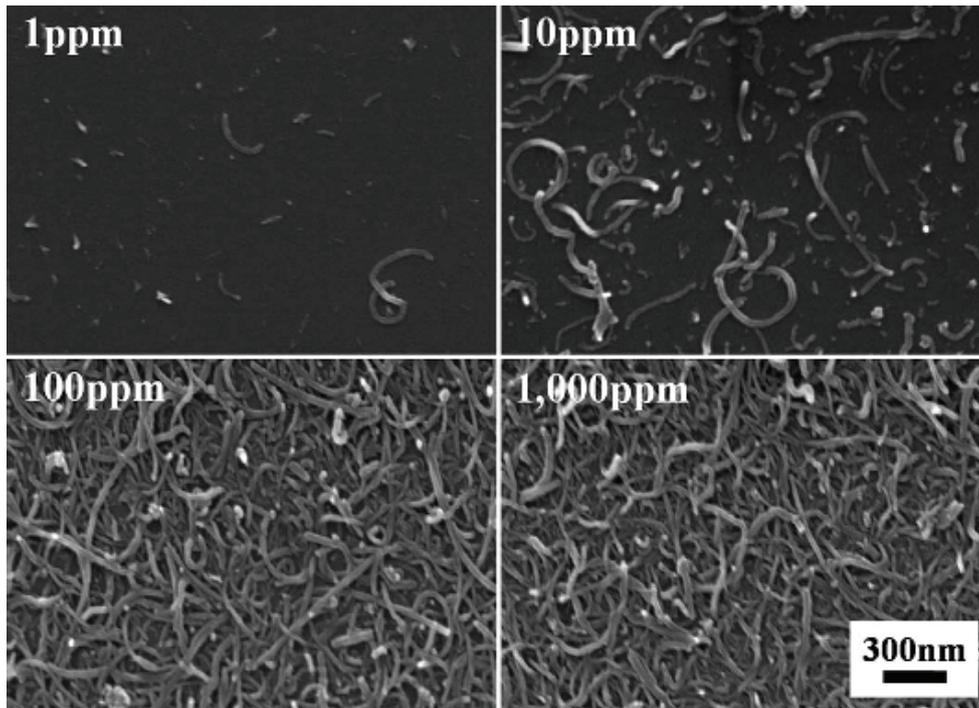


Fig. 1 SEM images of MWCNT adhering to collagen-coated cover glass treated with MWCNT suspensions at the concentrations of 1, 10, 100 and 1,000ppm for 3 hours

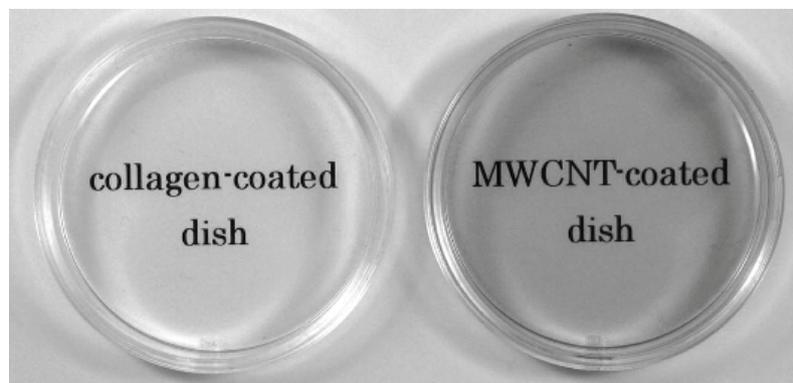


Fig. 2 Transparency of the MWCNT- and collagen-coated dishes

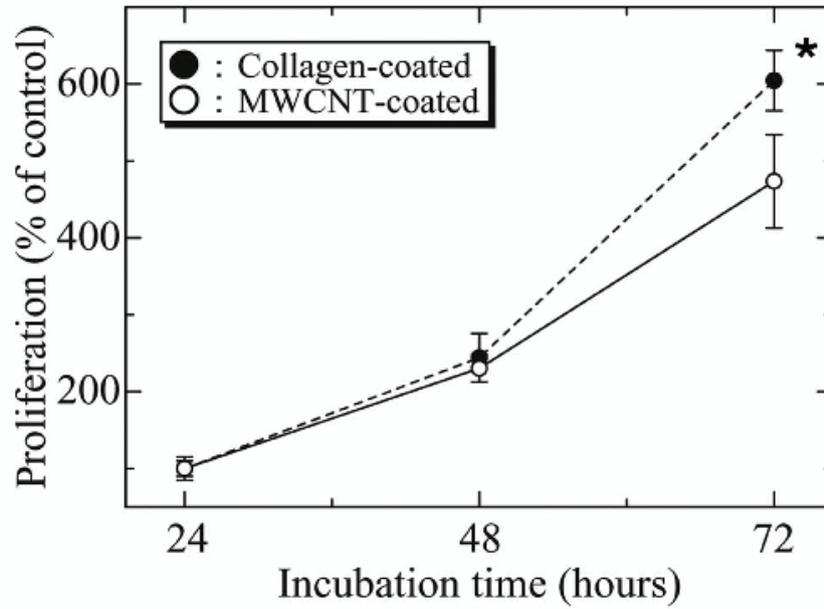


Fig. 3 Quantification of MC3T3-E1 cell growth on an MWCNT-coated dish

Values represent mean cell counts \pm SD from n=6 fields of observation per treatment; *, $p < 0.05$ compared with MWCNT-coated dish at 72 hours.

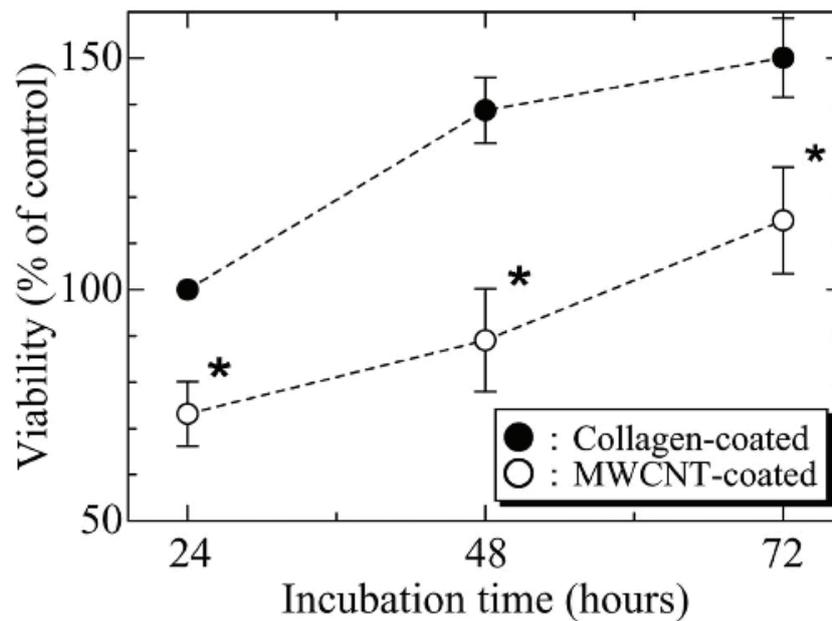


Fig. 4 Viability of MC3T3-E1 cells cultured on the MWCNT- and collagen-coated dishes

Values represent mean cell viability \pm SD from n=5; *, $p < 0.05$ compared with collagen-coated dish

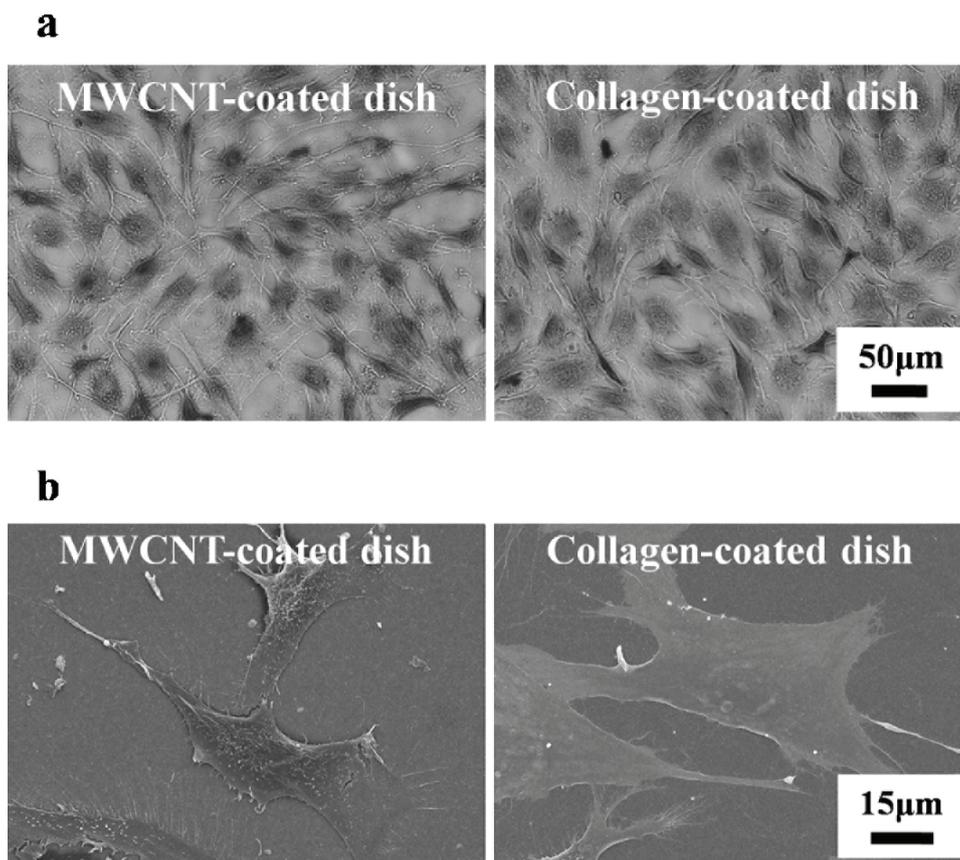


Fig. 5 (a) Optical microscope image of MC3T3-E1 cells on the surface of collagen- and MWCNT-coated dishes

(b) Low magnification SEM image of MC3T3-E1 cells on the surface of collagen- and MWCNT-coated dishes

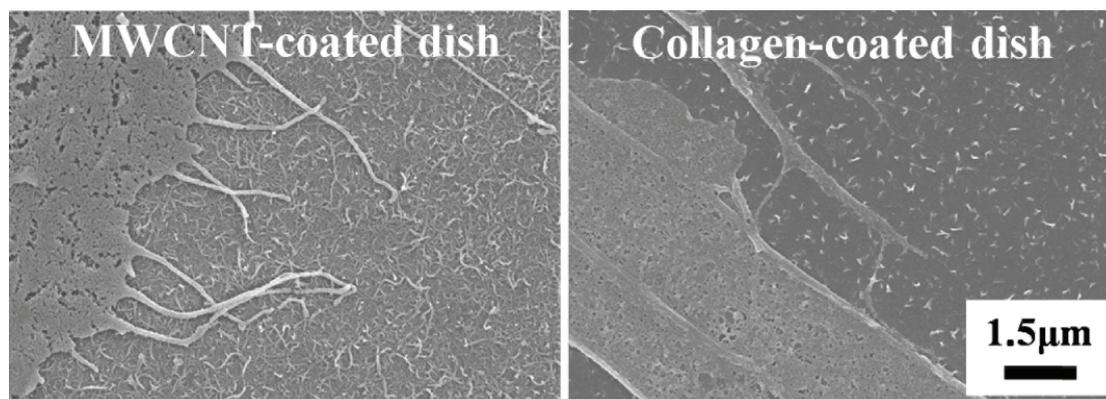


Fig. 6 High magnification SEM images of MC3T3-E1 cells on the surface of the collagen- and MWCNT-coated dishes

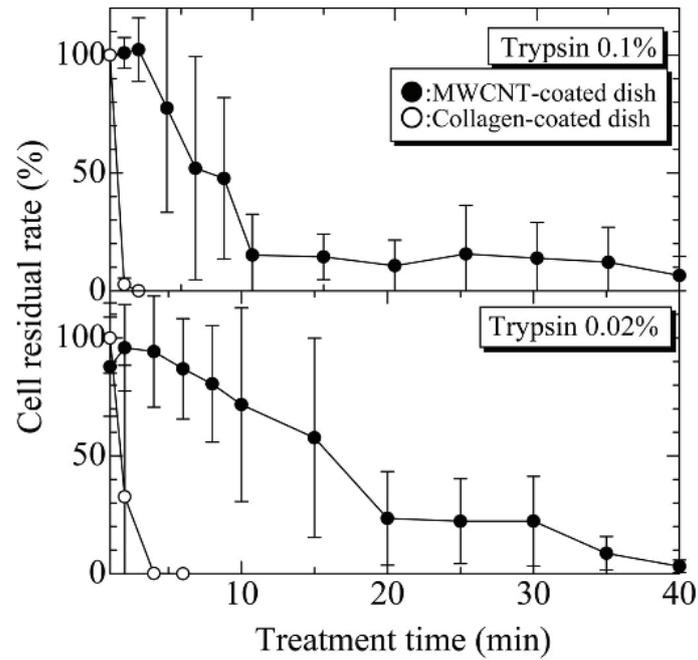


Fig. 7 Cell attachment test

○: Collagen-coated dish, ●: MWCNT-coated dish.

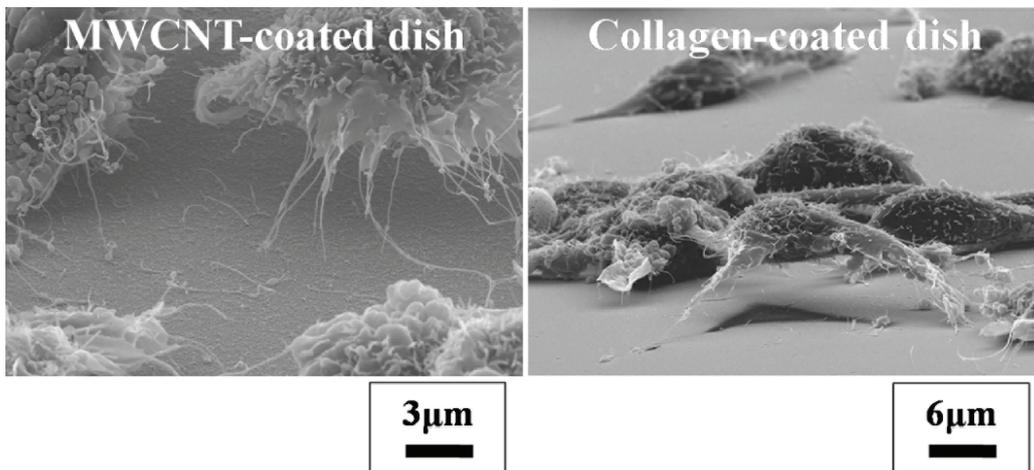


Fig. 8 SEM images of MC3T3-E1 cells treated with 0.02% Trypsin-EDTA for 1 to 2 minutes on the surface of a collagen- and MWCNTs-coated dish

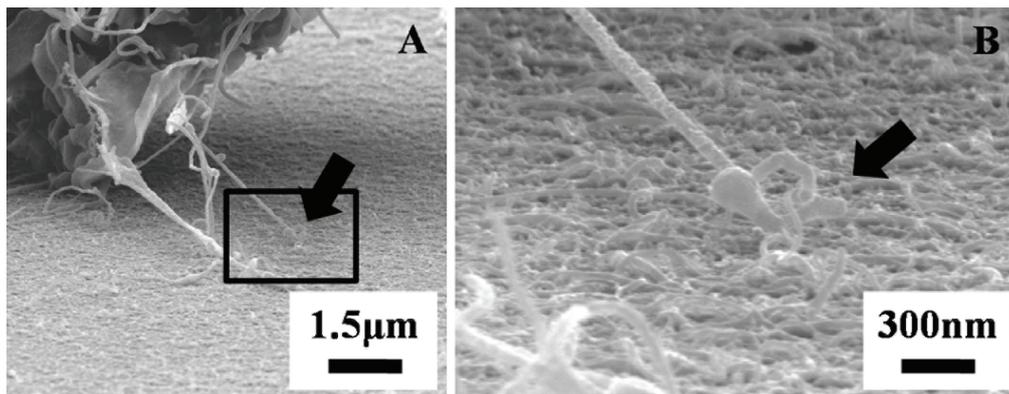


Fig. 9 SEM images of mechanical coupling of filopodia on an MWCNT-coated dish
A: low magnification view, B: enlargement of the square in A.
The arrow: mechanical coupling between filopodia and MWCNTs.