



Title	3D collagen scaffolds coated with multiwalled carbon nanotubes : Initial cell attachment to internal surface
Author(s)	Hirata, Eri; Uo, Motohiro; Nodasaka, Yoshinobu; Takita, Hiroko; Ushijima, Natsumi; Akasaka, Tsukasa; Watari, Fumio; Yokoyama, Atsuro
Citation	Journal of Biomedical Materials Research Part B: Applied Biomaterials, 93B(2), 544-550 <a href="https://doi.org/10.1002/jbm.b.31613">https://doi.org/10.1002/jbm.b.31613</a>
Issue Date	2010-05
Doc URL	<a href="http://hdl.handle.net/2115/43280">http://hdl.handle.net/2115/43280</a>
Type	article (author version)
File Information	Hirata_JBMR93B.pdf



[Instructions for use](#)

## **3D collagen scaffolds coated with multiwalled carbon nanotubes. —Initial cell attachment to internal surface**

Eri Hirata<sup>a</sup>, Motohiro Uo<sup>b</sup>, Yoshinobu Nodasaka<sup>c</sup>, Hiroko Takita<sup>c</sup>, Natsumi Ushijima<sup>c</sup>, Tsukasa Akasaka<sup>b</sup>, Fumio Watari<sup>b</sup> and Atsuro Yokoyama<sup>a</sup>

a. Department of Oral Functional Prosthodontics Division of Oral Functional Science, Graduate School of Dental Medicine, Hokkaido University, Kita 13, Nishi 7, Kita-ku, Sapporo 060-8586

b. Department of Biomedical, Dental Materials and Science, Graduate School of Dental Medicine, Hokkaido University, Kita 13, Nishi 7, Kita-ku, Sapporo 060-8586

c. Support Section for Education and Research, Graduate School of Dental Medicine, Hokkaido University, Kita 13, Nishi 7, Kita-ku, Sapporo 060-8586

### **Abstract**

The cell adhesion in a multiwalled carbon nanotube-coated collagen sponge (MWCNT-coated sponge) was investigated. Immediately after seeding, the cells adhered to the inner surface of the MWCNT-coated sponge, and a significantly larger number of cells were observed there than for a pure collagen sponge used as control. On the MWCNT-coated sponge, the cells appeared favorable adhesion and spread in the early stages in the center part of the sponge which cells rarely attached without MWCNT-coating. It was suggested that the physical structure of MWCNTs was effective for initial adhesion of cells from the result of serum-free culture. MWCNT-coating makes the material a suitable 3D scaffold for cell culturing, as opposed to other scaffold systems where such an effect is not seen.

### **1. Introduction**

Carbon nanotubes (CNTs) have attracted a great deal of attention for tissue engineering<sup>1-4</sup> because of their characteristic physical and biological properties and potential for a variety of applications<sup>5-8</sup>. For tissue engineering, many different three-dimensional (3D) cell culture scaffolds have been developed in order to gain more space for cell culture<sup>9</sup>, to build up the 3D structure of cells<sup>10,11</sup>, and for use in cell transplantation<sup>12</sup>. Collagen sponge honeycomb has already been used for many kinds of tissue engineering research<sup>13-15</sup> because of its high biocompatibility, large pore volume and pore diameter which appropriate for tissue and vessel invasion. However, the cells hardly attach to the surfaces of the internal pores of 3D scaffolds because almost all the cells pass through the pores during cell seeding<sup>10,16</sup>. In a previous study, we utilized the unique structure of multiwalled carbon nanotubes (MWCNTs) and

developed a 3D collagen scaffold coated with MWCNTs (MWCNT-coated sponge)<sup>17</sup>. MWCNTs homogeneously covered the surface of the collagen sponge without changing its 3D structure. Optical microscope observation showed that numerous cells adhered to the internal pores of the MWCNT-coated sponge in comparison with those of the collagen sponge alone. These results revealed that coating with MWCNTs improved the cell adhesion on the inside surface of the collagen sponge, and accordingly confirmed the effectiveness of coating with MWCNTs. In this study, we focused on the difference of cell adhesion in the central region between non-coated and MWCNT-coated sponge, and clarify the mechanism by which cells adhere especially in early stage of cultivation.

## 2. Materials & Methods

### 2.1. Materials characterization

The collagen sponge honeycomb (AteloCell<sup>®</sup>; 9mm in diameter, 2mm in thickness, Koken Co.,Ltd, Japan) was composed of a collagen membrane, 1  $\mu\text{m}$  in thickness, that had smooth surfaces. The sponge had parallel pores, 200-400 $\mu\text{m}$  in diameter, with a honeycomb structure extending from the top to the bottom. The surface of the collagen sponge was coated with the MWCNTs by the method described previously<sup>17</sup>. The MWCNT-coated sponge was observed by scanning electron microscopy (Hitachi S-4000, Japan) and transmission electron microscopy (Hitachi H-800, Japan).

### 2.2. Cell culture

Human osteosarcoma cell line Saos2 is widely used in studies on bone cell, adhesion, proliferation and differentiation<sup>18</sup>. Saos2 cells were suspended at  $1.0 \times 10^6$  cells/ml in Dulbecco's modified Eagle's medium (D-MEM; Sigma, France) containing 10% fetal bovine serum (FBS; Biowest, U.S.A.) and 1% penicillin/streptomycin. Collagen sponges and MWCNT-coated sponges were placed in the wells of a 48-well plate, and 100 $\mu\text{l}$  of the cell suspension was seeded on the scaffold in the wells and then incubated at 37°C in a 5% CO<sub>2</sub> atmosphere for each incubation period mentioned below.

### 2.3. SEM observation and measurement of cell number

After each incubation period, the samples were fixed with 2% glutaraldehyde, dehydrated and dried using the critical-point method. The central regions (380  $\mu\text{m}$  x 300  $\mu\text{m}$ ) of sectioned specimens were observed by SEM and the numbers of cells that adhered in the area was measured. Sixteen observation regions were randomly selected from the central parts of each section and the attached cell numbers in each SEM image were counted at 3 and 7 days. Then the difference between attached cell numbers on MWCNT-coated and uncoated collagen sponges was statistically analyzed with Student's two-tailed *t*-test. Statistically significant values were defined as  $p < 0.05$ . Moreover, the initial cell adhesion, especially the cell morphology just after attachment to the scaffolds and conducted observations at 15 and 60 minutes after seeding, was investigated.

## 2.4. Actin visualization

The cytoskeletal organization was determined by actin labeling with Rhodamine Phalloidin using F-Actin Visualization Biochem Kit (Cytoskeleton, Inc., U.S.A). After 1day and 7days incubation, whole sponges were fixed for 15 min and permeabilized for 10 min. After washing, the cells were incubated with Rhodamine Phalloidin for 30 min at room temperature. Then, the center part of the sponges were sliced and observed with an inverted microscope (Nikon Ti-E, Japan) with a confocal laser scanning system (CLSM, Nikon A1, Japan).

## 2.5. Cell attachment in serum-free medium

In order to estimate the effect of serum on the cell attachment, Saos2 cells ( $1 \times 10^7$  cells/mL) were suspended in Dulbecco's modified Eagle's medium (D-MEM; Sigma, France) with or without 10% fetal bovine serum (FBS; Biowest, U.S.A.). Each cell suspension was seeded to both sponges respectively. After 1 hour cultivation at 37°C in a 5% CO<sub>2</sub> atmosphere, the samples fixed, critical-point dried and counted the cell number on the center region using the above-mentioned SEM method.

# 3. Result

## 3.1. Materials characterization

SEM and TEM images of the MWCNT-coated sponge were shown in Figure 1. The honeycomb shape of the collagen sponge pores remained after coating with MWCNTs as shown (Figure 1a). Figure 1b shows that whole surface was homogeneously covered by MWCNTs without aggregation. TEM observations (Figure 1c and d) reveal that MWCNTs coated the collagen sponge surface as a monolayer and the thickness of the MWCNT coating estimated to be approximately 20-30 nm. MWCNTs maintained a unique tubular structure which looks like a sea-anemone on the surface of the collagen sponge.

## 3.2. Evaluation of attached cell density

First, to investigate the cell density and proliferation on the internal pore surface, the scaffolds were vertically sectioned along the pore direction at 3 and 7 days after incubation (Figure 2a). Figure 2b-e show SEM images of the cells that adhered to the inside wall of the center region of the sponge. Even after 7 days of incubation, there were few cells on the collagen sponge as shown in Figure 2d. In contrast, a large number of cells adhered to the whole surface of the MWCNT-coated sponge after 3 and 7 days of incubation as shown in Figure 2c and d. The cells on the collagen sponge increased from 3 to 7 days; however, their number of the cells was still smaller than on the MWCNT-coated sponge. There were significant differences in the numbers of cells on the sponges between with and without MWCNTs at 3 and 7 days shown in Figure 3.

## 3.3. Actin visualization

At 1 day, cells on the MWCNT-coated sponge spread more than on the collagen sponge and cytoplasmic meshwork was becoming apparent (Figure 4a, b). At 7 days, the stress fibers of actin were clearly observed in all cells on the center part of the CNT-coated sponge (Figure 4d), however those in the cells on the collagen sponge were still not clearly observed (Figure 4c).

### 3.4. Cell morphology in early stage

Figures 5a and 6a show that cells rarely attached on the collagen sponge at 15 and 60 min after cell seeding. Figure 5a was obtained with difficulty because there were hardly any attached cells on the scaffold at 15 min. The cells were still spherical and had attached to the collagen surface at few points of contact after 15 min (Figure 5c). In contrast, the cells adhered to the surface with a wide contact area and started to spread to the inside surface of the MWCNT-coated sponge (Figure 5b). In the high magnification shown in Figure 5d, numerous filopodia of the cell are entangled the MWCNTs. At 60 min, the cells that adhered tightly to the inside wall of the MWCNT-coated sponge appeared to spread as shown in Figure 6b and d, whereas the cells on the collagen sponge, as shown in Figure 6a and c, were still spherical and barely adhered to the inside surface with fewer filopodia than the cells on the MWCNT at 15 min.

### 3.5. Cell attachment in early stage with or without serum

Figure 7 shows the comparison of the cell numbers attached to the center part of each sponge with and without FBS (serum). Regardless of the serum addition, significantly larger number of cells was attached to the center part of the MWCNT-coated sponge than to the collagen sponge. The cells attached to center part of each sponge in serum-free medium as many as in medium with serum.

## 4. Discussion

There have been no reports of cells attaching and growing in the inside wall of the center region because even on the collagen sponge, cells hardly adhered to the inside and vertical pore surface just after seeding. McKegney et al. reported that a large number of cells were found in a collagen sponge with pores by confocal laser scanning microscopy examination<sup>13</sup>. In their case, maximum cell numbers were found at 10  $\mu\text{m}$  depth; however, maximum cell penetration was found at a depth of 120  $\mu\text{m}$ . In this study, we observed the surface structure of the MWCNT-coated sponge in detail and estimated the quantitative difference of cell adhesion to the inside wall of the center region of MWCNT-coated sponge. At 3days and 7days, significantly large number of cells adhered to the center part (at a depth of 1 mm from surface) of MWCNT-coated sponge. It is widely recognized that good initial cell attachment promotes further cellular proliferation<sup>19,20</sup>. Therefore, MWCNT-coated sponge would be helpful for the effective cell culture using whole pore surfaces of such 3D cell culture scaffolds because the efficient cells were entrapped on the whole surface. However the cell number did not increase between from 3 days to 7days. In general, when 3D cellular constructs are grown in static culture, cells on the outer surface of the constructs are typically viable and proliferate readily whereas cells within the construct may be less active or necrotic<sup>21</sup>. It was suggested that the dense cell growth occurred especially on the

outer surface of the sponge, but the cell on the center of the sponge were not well proliferated because more cells attached to whole surface of MWCNT-coated sponge homogeneously in the early stage of cultivation. Consequently, the way to improve the circulation of the medium in the pores such as perfusion culture would effect on the proliferation of the cells attached well on the center of the MWCNT-coated sponge.

In order to clarify the mechanism responsible for the improvement of proliferation induced by coating with MWCNTs, we focused on the cell morphology just after seeding. On the MWCNT-coated sponge the cells adhered and became spread out after short time such as 15 minutes whereas the cells attached at few point to the surface of collagen sponge. Moreover, a large number of actin would be formed on the MWCNT-coated sponge than that on the collagen sponge. Actin staining is popularly used to assess cell motility, cell spreading, cell shape and actin filaments<sup>22</sup>. Okumura et al. reported that the surface topography reflects the difference in organization of actin contributing factors in migration<sup>23</sup>. The superior formation of the actin in the cells on MWCNT-coated sponge could be related to the favorable cell migration on that surface. Therefore, MWCNT-coating could improve the cell attachment on the inside surface of the collagen sponge. After adhesion to the surface of the scaffold, the cells on the MWCNT-coated sponge could start extend rapidly in comparison with those on the collagen sponge and there was a larger number of cells on the inside surface of the MWCNT-coated sponge.

Cell adhesion to a biomaterial surface is a complex and dynamic process. Macromolecular complexes in serum play a role in mediating the material surface to extracellular matrix<sup>24</sup>. MWCNTs have high protein-absorption ability<sup>25</sup>. Various kinds of proteins involved in the serum would affect the chemical property of the MWCNT-coated surface. In order to estimate the effect of proteins in the serum, a serum-free culture was carried out. In spite of the serum-free culture, a significantly larger number of cells were attached to the center part of the MWCNT-coated sponge than to the collagen sponge. Therefore, the absorption of serum proteins on MWCNTs would not be necessary for cell adhesion, rather, one of the reasons suggested was that steric nanostructure of the MWCNT-coated surface, which looks like a sea-anemone, might be effective in entrapping the cell while the cells pass through the vertical wall of the sponge pore.

Several studies have examined 3D scaffolds with CNTs. Correa-Durate et al. fabricated a CNT-based 3D network scaffold without pores and confirmed cell growth, spreading, and adhesion<sup>26</sup>. Abarrategi et al. reported that cells grew well on MWCNT/CHI scaffolds with pores<sup>27</sup>. However, the cell attachment and growth occurred mainly on the surface of the outside of the scaffold and outer pores and seldom penetrated into the internal pores of the scaffolds containing MWCNTs. In this study, the MWCNT coating on the whole collagen sponge surface improved the cell attachment to the inside surfaces of the sponge pores, so that homogeneous cell adhesion on the whole sponge surface was achieved.

The MWCNT-coated sponge is the 3D scaffold for cell culturing with such an effect which the other scaffolds do not have.

#### 4. Conclusion

MWCNT-coating improved cell adhesion of collagen sponge even on the vertical and smooth collagen surface to which the cells hardly attach. The physical structure of MWCNT-coated surface could affect the entrapment of the cells during the cell seeding. The MWCNT-coated sponge with favorable characteristic of the cell adhesion is suitable for 3D cell culture and will be useful for tissue engineering such as cell transplantation.

**ACKNOWLEDGMENT** This work was supported by a Grant-in Aid for Basic Research (B) 19390488 from the Ministry of Education, Science, Culture and Sport of Japan and Iwadare Scholarship Foundation. Confocal images were acquired in the Nikon Imaging Center at Hokkaido University.

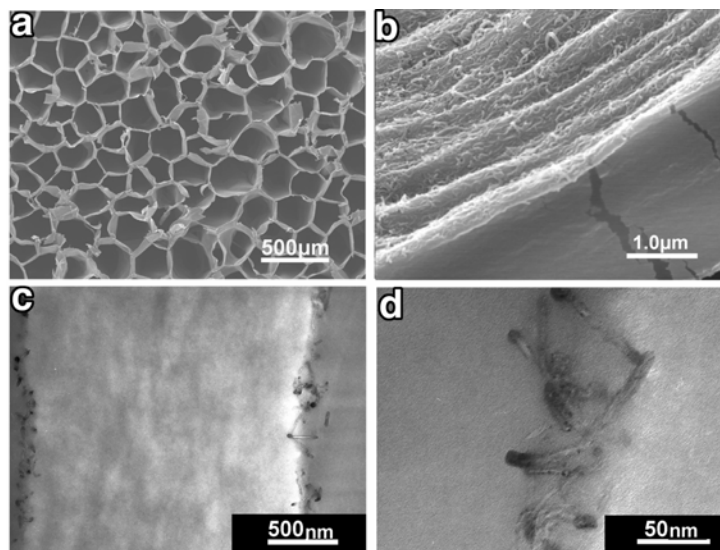
#### REFERENCES

1. Zanello LP, Zhao B, Hu H , Haddon RC. Bone cell proliferation on carbon nanotubes. *Nano Lett.* 2006; 6:562-567.
2. Aoki N, Akasaka T, Watari F, Yokoyama A. Carbon Nanotubes as Scaffolds for Cell Culture and Effect on Cellular Functions. *Dental Material Journal* 2007; 26: 178-185.
3. Kam NW, Jan E , Kotov NA. Electrical Stimulation of Neural Stem Cells Mediated by Humanized Carbon Nanotube Composite Made with Extracellular Matrix protein. *Nano Lett.* 2009; 9: 273-278.
4. Edwards SL, Church JS, Werkmeister JA , Ramshaw JA. Tubular micro-scale multiwalled carbon-based scaffolds for tissue engineering *Biomaterials* 2009; 30: 1725-1731.
5. Sato Y, Yokoyama A, Kasai T, Hashiguchi S, Ootsubo M, Ogino S, Sashida N, Namura M, Motomiya K, Jeyadevan B, Tohji K. In vivo rat subcutaneous tissue response of binder-free multi-walled carbon nanotube blocks cross-linked by de-fluorination. *Carbon* 2008; 46: 1927-3194.
6. Yokoyama A, Sato Y, Nodasaka Y, Yamamoto S, Kawasaki T, Shindoh M, Kohgo T, Akasaka T, Uo M, Watari F, Tohji K. Biological Behavior of Hat-Stacked Carbon Nanofibers in the Suctaneous Tissue in Rats. *Nano Lett.* 2005; 5: 157-161.
7. Akasaka T, Watari F. Capture of bacteria by flexible carbon nanotubes. *Acta Biomater* 2009; 5: 607-612.
8. Akasaka T, Watari F, Sato Y, Tohji K. Apatite formation on carbon nanotubes. *Mater Sci Eng C* 2006; 26: 675-678.

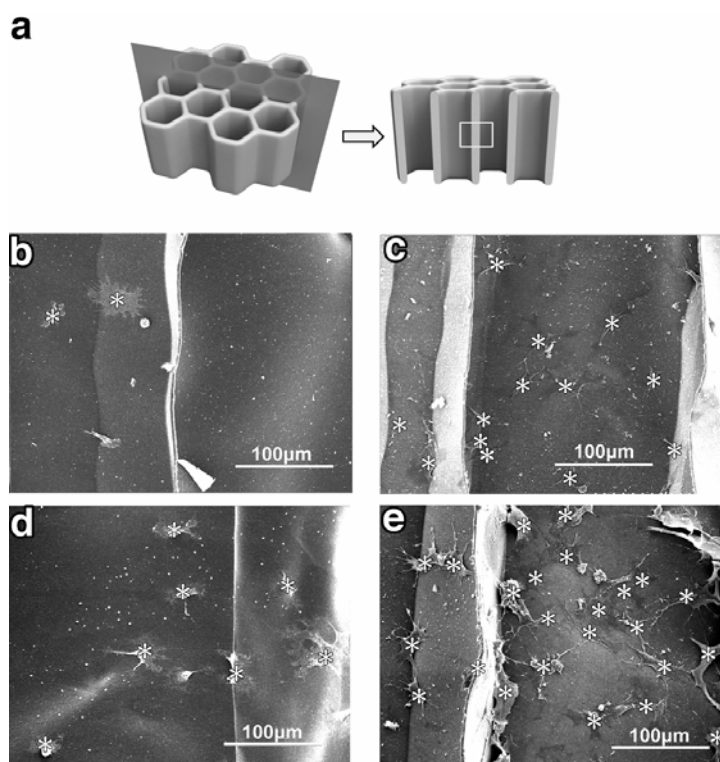
9. Shimizu K, Ito A, Honda H. Enhanced Cell-Seeding into 3D Porous Scaffolds by Use of Magnetite Nanoparticles. *J Biomed Mater Res B Appl Biomater* 2006; 77: 265-272.
10. Holy CE, Shoichet MS, Davies JE. Engineering three-dimensional bone tissue in vitro using biodegradable scaffolds: Investigating initial cell-seeding density and culture period *J Biomed Mater Res* 2000; 51: 376-382.
11. Rezwani K, Chen QZ, Blaker JJ, Boccaccini AR. Biodegradable and bioactive porous polymer/inorganic composite scaffolds for bone tissue engineering. *Biomaterials* 2006; 27: 3413-3431.
12. Tomita M, Lavik E, Klassen H, Zahir T, Langer R, Young M J. Biodegradable Polymer Composite Grafts Promote the Survival and Differentiation of Retinal Progenitor Cells. *Stem Cells* 2005; 23: 1579-1588.
13. Rodriguez AP, Missana L, Nagatsuka H, Gunduz M, Tsujigiwa H, Rivera R, Nagai N. Efficacy of atelocollagen honeycomb scaffold in bone formation using KUSA/A1 cells. *J Biomed Mater Res A* 2006; 77: 707-717.
14. George J, Onodera J, Miyata T. Biodegradable honeycomb collagen scaffold for dermal tissue engineering. *J Biomed Mater Res A* 2008; 80: 1103-1111.
15. Kakudo N, Shimotsuma A, Miyake S, Kushida S, Kusumoto K. Bone tissue engineering using human adipose-derived stem cells and honeycomb collagen scaffold. *J Biomed Mater Res A* 2008; 84: 191-197.
16. Mckgney M, Taggart I, Grant MH. The influence of crosslinking agents and diamines on the pore size, morphology and the biological stability of collagen sponges and their effect on cell penetration through the sponge matrix. *J Mater Sci-Mater M* 2001; 12: 833-844.
17. Hirata E, Uo M, Takita H, Akasaka T, Watari F, Yokoyama A. Development of a 3D Collagen Scaffold Coated With Multiwalled Carbon Nanotubes. *J Biomed Mater Res B Appl Biomater* 2009; 90B: 629-634.
18. Rodan SB, Imai Y, Thiede MA, Wesolowski G, Thompson D, Bar-Shavit Z, Shull S, Mann K, Rodan GA. Characterization of a Human Osteosarcoma Cell Line (Saos-2) with Osteoblastic Properties. *Cancer Res* 1987; 47: 4961-4966.
19. Susan L I, Genevieve M C, Michael J Y and Antonios G M. Three-dimensional culture of rat calvarial osteoblasts in porous biodegradable polymers. *Biomaterials* 1998; 15: 1405-1412.
20. Ingber DE. Tensegrity II. How structural networks influence cellular information processing networks. *J Cell Sci* 2003; 116: 1397-1408.



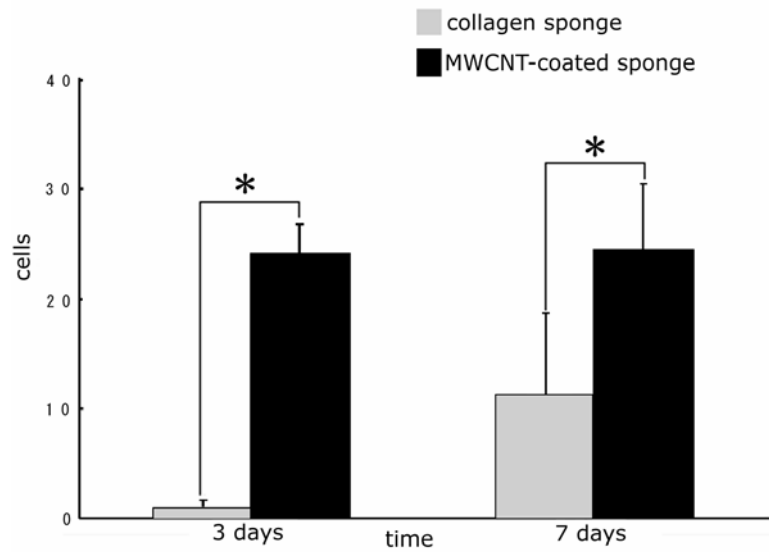
21. Okumura A, Goto M, Goto T, Yoshinari M, Masuko S, Takeshi K, Tanaka T. Substrate affects the initial attachment and subsequent behavior of human osteoblastic cells (Saos-2). *Biomaterials* 2001; 22: 2263-2271.
22. Ben-Ze'ev A, Farmer SR, Penman S. Protein Synthesis Requires Cell-surface contact while nuclear Events Respond to Cell Shape in Anchorage-Dependent Fibroblast. *Cell* 1980; 21: 365-372.
23. Malik MA, Puleo DA, Bizios R, Doremus RH. Osteoblasts on hydroxyapatite, alumina, and bone surfaces *in vivo* morphology during the first 2h of attachment. *Biomaterials* 1992; 13: 123-128.
24. Howlett CR, Evans MDM, Walsh WR, Johnson G, Steele JG. Mechanism of initial attachment of cells derived from human bone to commonly used prosthetic materials during cell culture. *Biomaterials* 1994; 13: 213-222.
25. Chen X, Tam UC, Czapinski JL, Lee GS, Rabuka D, Zettl A, Bertozzi CR. Interfacing Carbon Nanotubes with Living Cells. *J. Am. Chem. Soc.* 2006; 128: 6292-6293.
26. Correa-Duarte MA, Wagner N, Rojas-Chapana J, Morszeck C, Thie M, Giersig M. Fabrication and Biocompatibility of Carbon Nanotube-Based 3D Networks as Scaffolds for Cell Seeding and Growth. *Nano Lett* 2004; 4: 2233-2236.
27. Abarrategi A, Gutierrez MC, Moreno-Vincente C, Hortiguela MJ, Ramos V, Lopez-Lacomba J, Ferrer ML, Monte F. Multiwall carbon nanotube scaffolds for tissue engineering purposes. *Biomaterials* 2008; 29: 94-102.



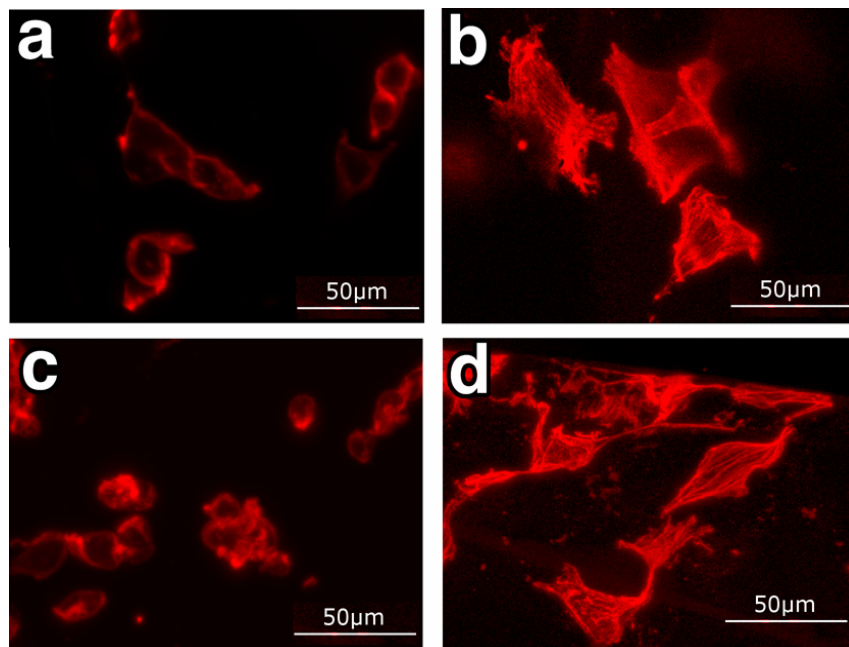
**Figure 1.** MWCNT-coated sponge. (a) SEM image of MWCNT-coated sponge. (b) SEM image of a cross section. (c) TEM image of a cross section. (d) High magnification of c.



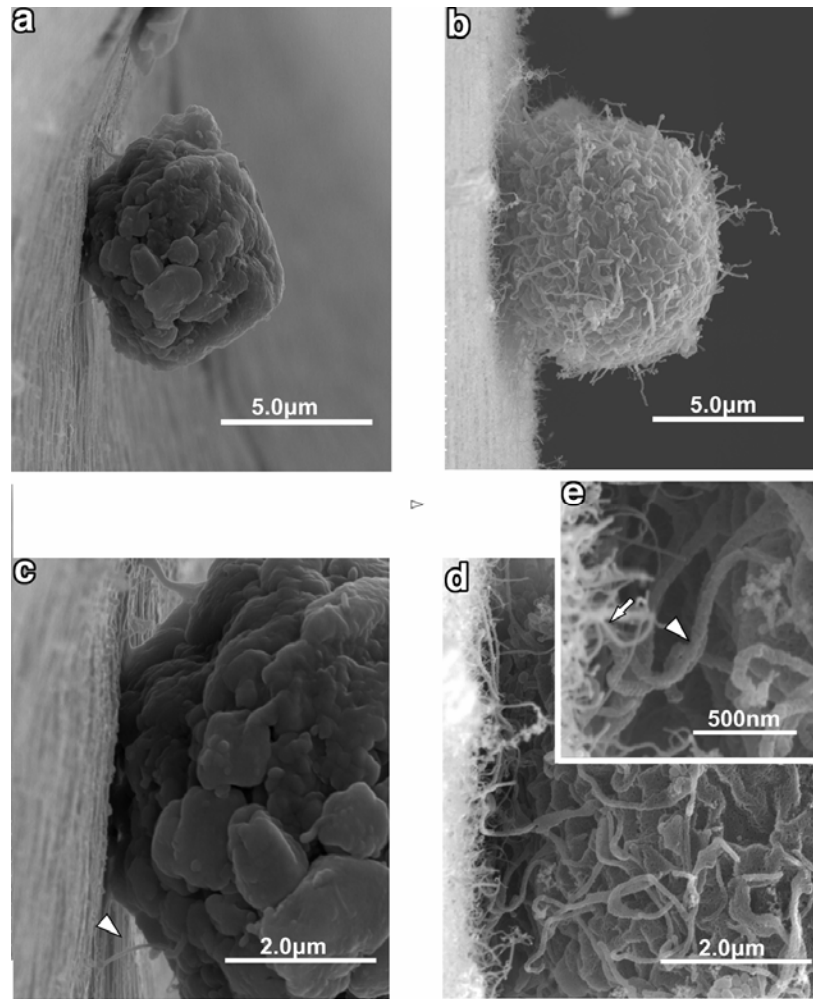
**Figure 2.** The proliferation of the cells adhering to the center part of each sponge at 3 days and 7 days. Every cell is marked by a white asterisk. (a) Diagram showing the method to slice (left) and the part inside the square used for SEM observation (right). (b) Few cells are observed on the collagen sponge at 3 days. (c) More cells are observed on the MWCNT-coated sponge than on the collagen sponge at 3 days. (d) A few more cells are observed on the collagen sponge at 7 days than at 3 days. (e) More cells adhere to the MWCNT-sponge.



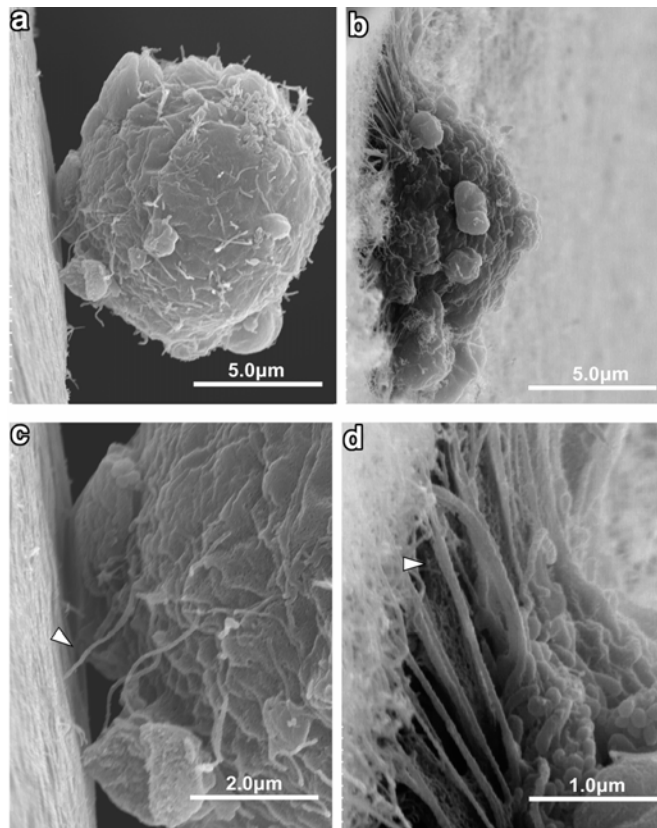
**Figure 3.** Quantification of the total number of cells per SEM image. Significant differences were found between the collagen sponge and MWCNT-coated sponge (\*) at 3 days and 7 days,  $p < 0.05$ .



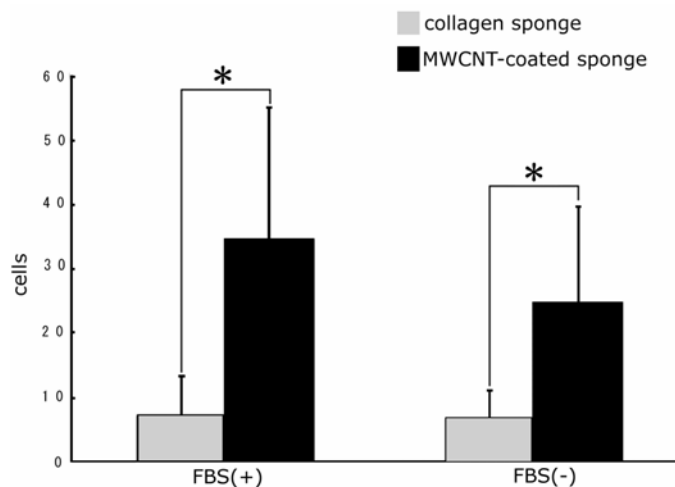
**Figure 4.** CLSM images of immunostained actin in Saos2 cells cultured on collagen sponge (a,c) and on MWCNT-coated sponge (b,d) after 1day (a,b) and 7days (c,d).



**Figure 5.** The morphology of the cell attachment to the inside surface of each sponge at 15 min after seeding. (a) The cell is hardly attached to the collagen sponge. (b) The cell adheres to the MWCNT-coated sponge. (c) High magnification of a. A few filopodia (white arrowhead) touch the surface of the collagen sponge. (d) High magnification of b. Many filopodia elongate to the MWCNTs. (e) Detail of the interface between MWCNTs and cell filopodia. The filopodia (white arrowhead) are entangled in the MWCNTs (white arrow).



**Figure 6.** The morphology of the cell attachment to the inside surface of each sponge at 60 min after seeding. (a) The still spherical cell has started to attach to the collagen sponge. (b) The cell spread on the MWCNT-coated sponge. (c) High magnification of a. A few filopodia (white arrowhead) elongate to the surface of the collagen sponge. (d) High magnification of b. Many more filopodia elongate (white arrowhead) to and are entangled in the MWCNTs.



**Figure 7.** Quantification of the total number of cells attached to each scaffolds at 60 min after seeding with or without serum. There are no significant differences between with and without serum,  $p < 0.05$ .