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

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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 engineering¹⁻⁴ because of their characteristic physical and biological properties and potential for a variety of applications⁵⁻⁸. For tissue engineering, many different three-dimensional (3D) cell culture scaffolds have been developed in order to gain more space for cell culture⁹, to build up the 3D structure of cells^{10,11}, and for use in cell transplantation¹². Collagen sponge honeycomb has already been used for many kinds of tissue engineering research¹³⁻¹⁵ 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^{10,16}. 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)¹⁷. 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[®]; 9mm in diameter, 2mm in thickness, Koken Co.,Ltd, Japan) was composed of a collagen membrane, 1 μm in thickness, that had smooth surfaces. The sponge had parallel pores, 200-400 μm 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 previously¹⁷. 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 differentiation¹⁸. Saos2 cells were suspended at 1.0×10^6 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 μl of the cell suspension was seeded on the scaffold in the wells and then incubated at 37°C in a 5% CO₂ 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 μm x 300 μm) 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 (1×10^7 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% CO₂ 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 examination¹³. In their case, maximum cell numbers were found at 10 μm depth; however, maximum cell penetration was found at a depth of 120 μm . 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^{19,20}. 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 necrotic²¹. 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 filaments²². Okumura et al. reported that the surface topography reflects the difference in organization of actin contributing factors in migration²³. 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 matrix²⁴. MWCNTs have high protein-absorption ability²⁵. 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 adhesion²⁶. Abarrategi et al. reported that cells grew well on MWCNT/CHI scaffolds with pores²⁷. 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.

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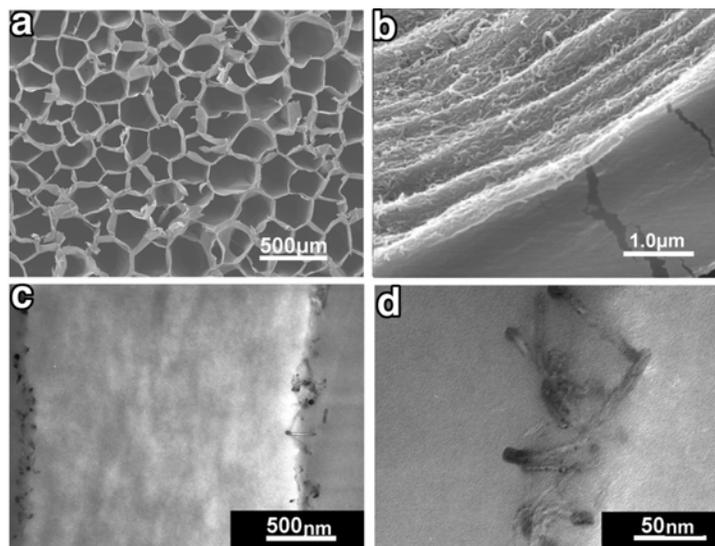


Figure 1. MWCNT-coated sponge. (a) SEM image of MWCNT-coated sponge. (b) SEM image of a cross section. (c) TEM image of a cross section. (d) High magnification of c.

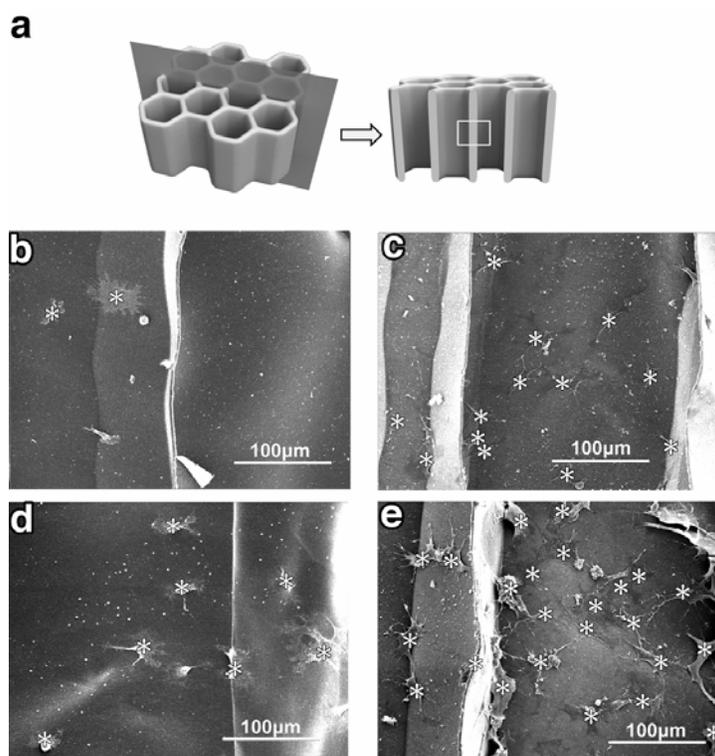


Figure 2. The proliferation of the cells adhering to the center part of each sponge at 3 days and 7 days. Every cell is marked by a white asterisk. (a) Diagram showing the method to slice (left) and the part inside the square used for SEM observation (right). (b) Few cells are observed on the collagen 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.

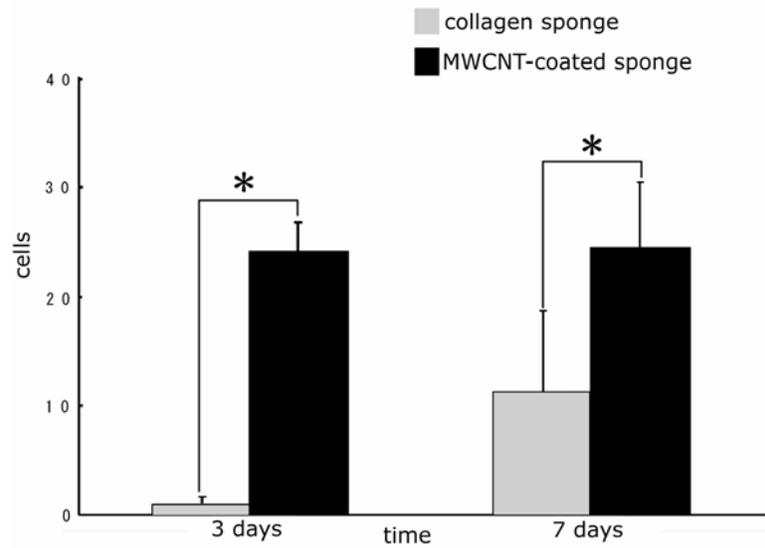


Figure 3. Quantification of the total number of cells per SEM image. Significant differences were found between the collagen sponge and MWCNT-coated sponge (*) at 3 days and 7 days, $p < 0.05$.

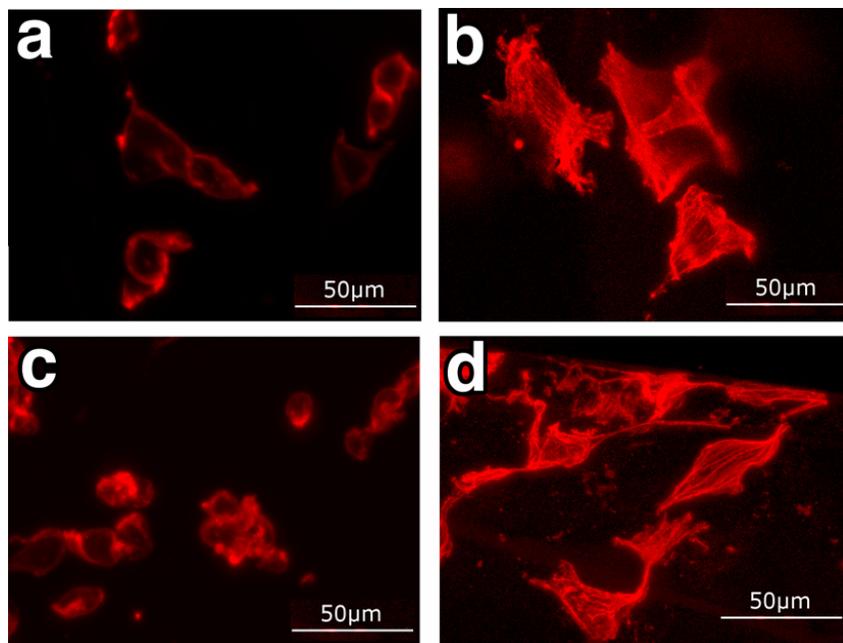


Figure 4. CLSM images of immunostained actin in Saos2 cells cultured on collagen sponge (a,c) and on MWCNT-coated sponge (b,d) after 1day (a,b) and 7days (c,d).

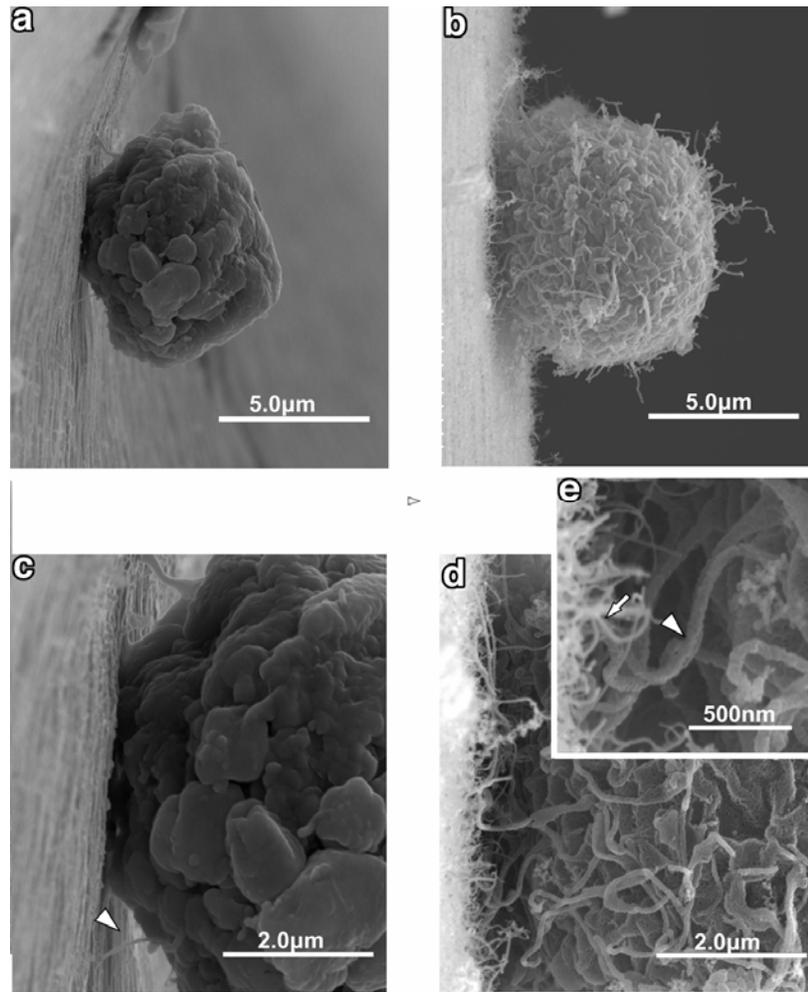


Figure 5. The morphology of the cell attachment to the inside surface of each sponge at 15 min after seeding. (a) The cell is hardly attached to the collagen sponge. (b) The cell adheres to the MWCNT-coated sponge. (c) High magnification of a. A few filopodia (white arrowhead) touch the surface of the 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).

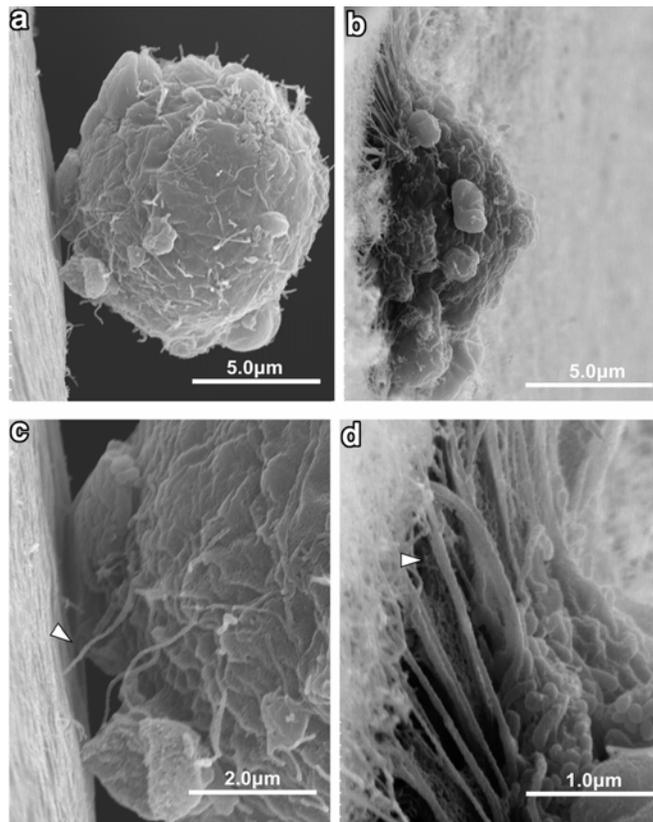


Figure 6. The morphology of the cell attachment to the inside surface of each sponge at 60 min after seeding. (a) The still spherical cell has started to attach to the collagen sponge. (b) The cell spread on the MWCNT-coated sponge. (c) High magnification of a. A few filopodia (white arrowhead) elongate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

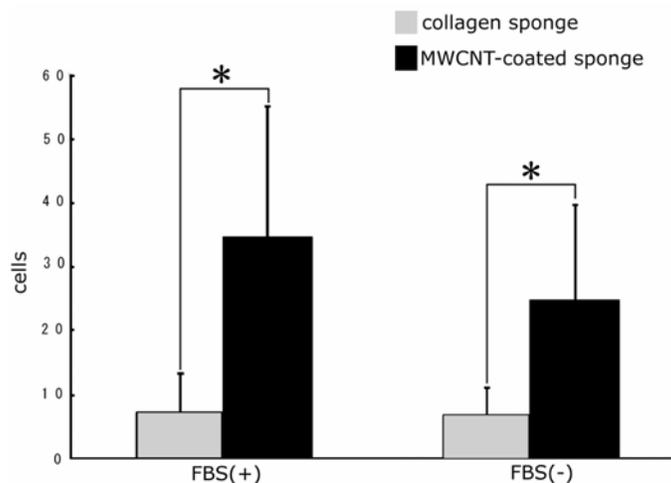


Figure 7. Quantification of the total number of cells attached to each scaffolds at 60 min after seeding with or without serum. There are no significant differences between with and without serum, $p < 0.05$.