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Fibroblast cell behavior on chemically functionalized carbon nanomaterials

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ABSTRACT

This paper describes the interaction of 3T3 fibroblast cells with chemically functionalized carbon nanomaterial substrates. Multiwall carbon nanotubes and carbon nanofibers were functionalized using acid treatment. A more hydrophilic surface was obtained by adding functional groups which are defects at the ends of the multi-walled carbon nanotubes and carbon nanofibers. 3T3 fibroblast cells were cultured on both as-grown and functionalized carbon nanomaterials on a glass slide. Visual differential interference contrast (DIC) images and fluorescent images of stained α, β tubulin showed the effectiveness of the functionalized nanomaterials as substrate materials. Good adhesion of the 3T3 fibroblast cells to the functionalized nanomaterials was verified and indicates that nanomaterials have potential for tissue scaffold development. No significant toxic effects of the nanomaterials on the 3T3 cells were observed. Possible explanations of why cells grow on nanomaterials are given.

1. Introduction

Carbon Nanotubes (CNTs) were discovered seventeen years ago and have attracted tremendous interest from many scientists in the fields of physics, chemistry, biomedicine, and materials science. Among these fields, biomedical applications of nanotechnology have an especially large potential. Three applications of Bio-Nanotechnology can be categorized as; (i) biomedical diagnostic techniques [1–6], (ii) drugs [3], and (iii) prostheses and implants [5]. Biomedical applications [4,6] for use outside of the body, such as diagnostic sensors like DNA, RNA chips, Lab-on-a-chip, and micro-Total Analysis Systems (μTAS) are suitable for analyzing bio-molecular samples. For inside the body, anticancer imaging and drugs, bio-MEMS devices, and gene therapy are major growing area. Other researchers [5] are working on prostheses and implants that include nanostructured materials. Tissue engineering based on nanotechnology is a particularly promising area since carbon nanotubes seem biocompatible as scaffolds for restoring and reinforcing damaged tissue [7,8]. Excellent mechanical properties with light weight are useful properties for developing strong 3D-scaffolds. However, cell culture studies must be carefully performed in order to take advantage of CNT properties for cell scaffolds. The studies should include proper wetting of nanomaterials, the interaction between cells and nanomaterials, growth factor behavior in extra cellular matrices (ECM), and cytotoxicity [9].

This paper describes fibroblast cell behavior on the surface of functionalized carbon nanomaterials. Two different types of carbon nanoscale materials are considered; carbon nanofibers (CNFs), and multi-walled carbon nanotubes (MWCNTs). CNFs are typically 150 nm in diameter and microns long while MWCNTs are 20 nm in diameter and mm long. High density MWCNT arrays were synthesized using a metallic catalyst, $Fe/Al_2O_3/SiO_2$, on Si wafers for the cell culturing experiments. MWCNT can grow up to 18 mm tall by water-assisted CVD. The mm long MWCNT array is easily peeled off the Si substrate, functionalized, and dispersed in dimethylformamide (DMF). The CNFs are commercially available and were purchased from Applied Sciences Inc.

The 3T3 cell line is a standard fibroblast cell line used in a wide spectrum of research and industrial biomedical applications to study important cell functions such as maintenance of cell shape, cell movement, and proliferation. In particular, cytoskeleton components such as tubulins play an important role in cell division and growth. Therefore the interaction of 3T3 cells with carbon nanotube will be important to study. In this paper, 3T3 fibroblast cells were cultured on functionalized CNF and MWCNT-coated substrates and stained with α, β tubulin. Nanotube-fibroblast cell interactions were then analyzed based on microtubule fluorescence and DIC images.

2. Materials and method

2.1. Synthesis of multi-walled carbon nanotubes

P-type Si wafers <100> 4 in. diameter with a 500 nm thick SiO_2 layer on the top and a typical resistivity of 1–20 Ω cm were used to grow the carbon nanotubes. An E-beam evaporator is used to deposit an Al thin film 10 nm thick on the SiO_2. Then Al is oxidized to form Al_2O_3. Finally, catalytic iron films of controlled thickness between 1 and 2 nm are deposited on the Al_2O_3 surface. The final substrate

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configuration is $Fe/Al_2O_3/SiO_2/Si$. The evaporation is carried out at a pressure of about 5×10^{-7} Torr. The final substrate is cut into a 5×5 mm size wafers. Various types of masks were used to make 2-dimensional patterns on the substrate including Fe patterned in 1 mm$\times$1 mm square blocks with a100 μm space between the blocks.

Nanotube array synthesis by thermal CVD was carried out in a horizontal 2 in. diameter tube furnace, the EasyTube™ model ET1000 produced by First Nano, Inc. The furnace has four mass flow controllers, and a vapor delivery system. Argon is used as the carrier gas to carry the water vapor to the reaction chamber. Argon is used to purge the reactor for 20 min while the CVD furnace is heated to 750 °C. Gas flow is then switched to ethylene, water, and hydrogen for the desired time for the deposition and CNT growth. After the nanotube array is synthesized, the array is cooled to ambient temperature which completes the last process step. During the cooling process, ethylene, water, and hydrogen flow are stopped and the system is purged with argon to prevent back flow of air from the exhaust line. The synthesized CNT arrays were characterized by environmental scanning electron microscopy (ESEM) and transmission electron microscopy (TEM). First, the CNT arrays are peeled off the substrate and dispersed in dimethylformamide (DMF) using bath ultrasonication without any purification steps. TEM samples were prepared by placing a small droplet of the dispersion onto a formvar-free lacy copper TEM grid and dried in air. A JEOL JEM-1230 TEM operated at120 KV is used for imaging.

Fig. 1(a,b) shows the ESEM results for the water-assisted growth conditions, which are: 200 SCCM of H_2, 200 SCCM of C_2H_4, and 750 °C growth temperature. With the increase of growth time up to 6 h, the length of CNT arrays keeps increasing. This CNT array has high density without impurities, other than amorphous carbon. The low impurities

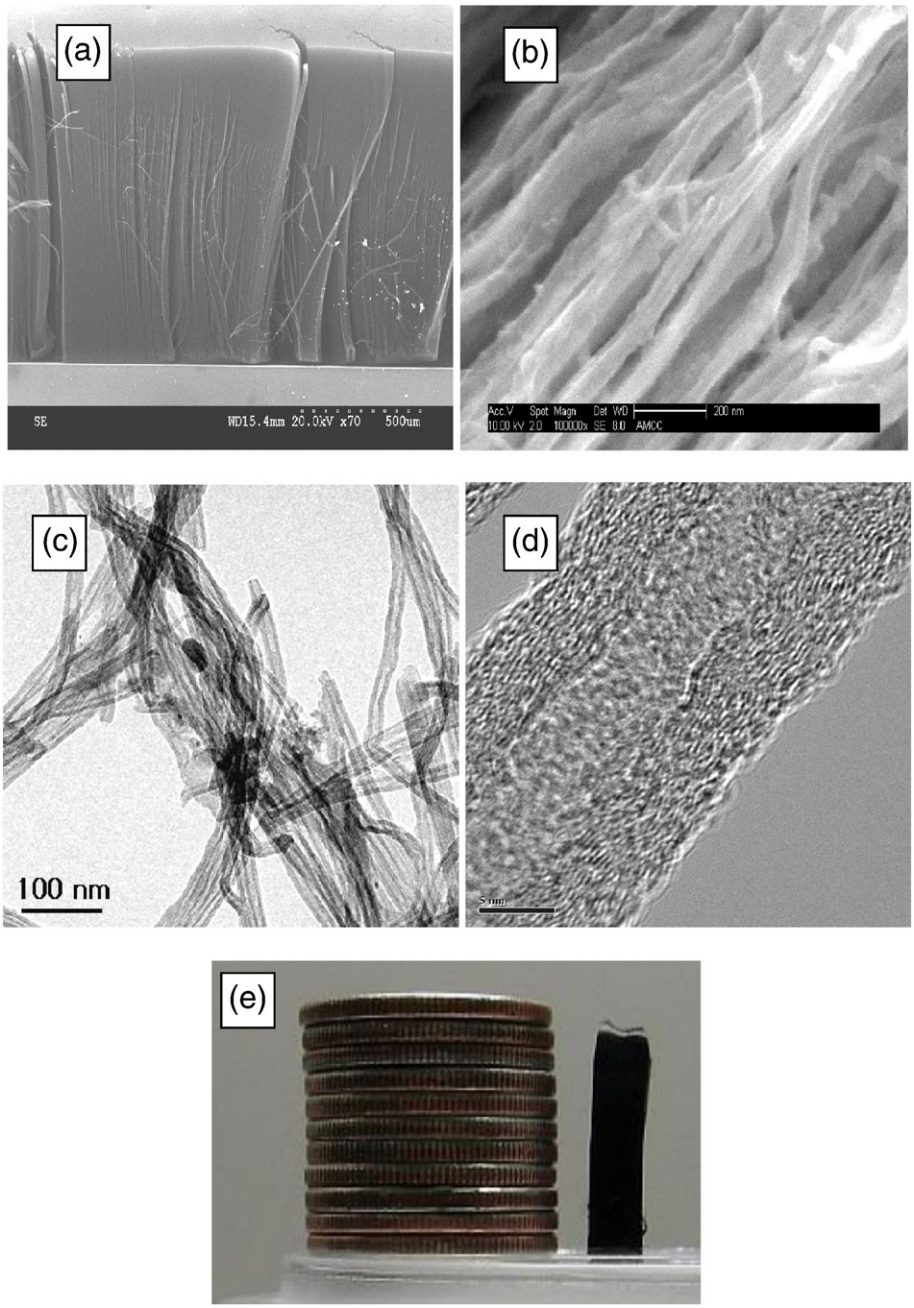

Fig. 1. ESEM images of aligned MWCNT arrays at: (a) low resolution, and (b) high resolution, HRTEM images at (c) low resolution, and (d) high resolution; (e) picture of a MWCNT array with 11 US quarters for size reference. Growth conditions: 200 SCCM of H_2 flow, 200 SCCM of C_2H_4 flow, 100 SCCM Bubbler flow, 750 °C growth temperature.

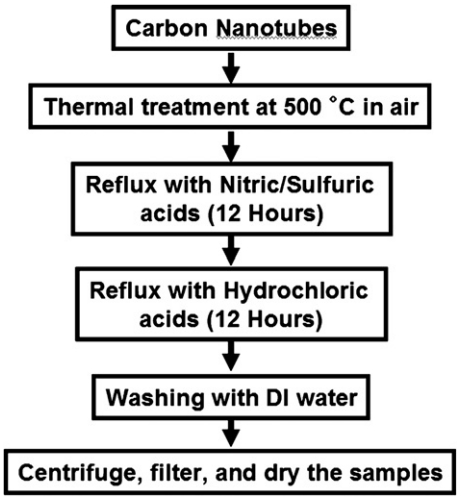

Fig. 2. Functionalization steps for MWCNTs and CNFs for use in cell culturing.

make the array ideal for further application development. Adhesion to the substrate is weak, and the CNT array is easily peeled off. Fig. 1(b) is the ESEM image of the aligned multi-wall carbon nanotube patterned array showing the alignment with high density. Fig. 1(c) shows the TEM images of nanotube array with lower magnification illustrating high quality of nanotubes without any metal catalyst particles and little amorphous carbon. Fig. 1(d) shows the high resolution TEM image showing that MWCNTs have 15–20 nm outer diameter and 5–8 nm inner diameter with 15 walls on average. As shown in Fig. 1(c), the diameters of the nanotubes are uniform without much impurity. Fig. 1(e) shows a tall MWCNT array on the Si wafer and US quarters for size reference. The nanotube average diameter is 20 nm and the aspect ratio (length/diameter) is 900,000:1. In order to measure the resistivity, epoxy was cast over a nanotube tower in a mold, both ends of the nano-composite tower were polished, and a wire was connected to the electrode using conductive epoxy. The resistivity of the 1 mm square block was 0.11 Ω cm. The resistivity of the individual nanotubes is in the range of about 10^{-3} to 10^{-4} Ω cm depending on the length, diameter, and processing of the nanotube. This shows that the nanotubes will form an electrically conductive scaffold for culturing cells.

2.2. Functionalization of CNF and MWCNT

Functionalization of CNF or nanotubes is to attach special chemical groups to the individual nanotubes that can act as receptors for other molecules or polymers. Functionalization can also improve the dispersion of nanotubes. The functionalization and dispersion procedures are shown in Fig. 2. An as-grown MWCNT array is first annealed at 500 °C for 2 h. The MWCNT array is then immersed in concentrated 3:1H_2SO_4/70% HNO_3 and reflux for 12 h. At this stage, the MWCNT array separates from the Si wafer automatically. Then 60% HCl was added to the acid mixture to facilitate the termination of the opened ends and the defects in the sidewalls of the MWCNTs with carboxylic acid groups. After 2 h refluxing the MWCNT solution, the MWCNT is filtered and washed with 0.1 M NaOH solution for neutralization. Then the MWCT is washed with distilled water 5 times and dried at 120 °C for 2 h. The MWCTN is dispersed in dimethylformamide (DMF, Fisher) by mechanical mixing and sonication for 2 h. Finally, the DMF–MWCNT solution is deposited on a glass slide and dried in a vacuum oven for 5 h. The final nanotube substrate is sterilized by ultra-violet light. The same procedure is used for CNF functionalization (PR-24, Applied Science Company). The CNF is used as received without further purification.

As shown in Fig. 3, 10 μm of water is placed on the surface of a 1 mm long high density nanotube array. Results show the super-hydrophobic surface behavior with more than 150 ± 5° contact angle. However, after functionalization, the nanotube array shows a more hydrophilic surface behavior with a 50° ± 5 contact angle. Probably, this hydrophilic surface might increase the adhesion and spreading of cells onto the nanotubes.

Fig. 4(a) shows the TEM image of MWCNT with clearly opened ends after functionalization. Fig. 4(b) shows environmental scanning electron microscope (ESEM) images of CNFs which show a chopped structure with opened ends after functionalization.

2.3. 3T3 fibroblast cells and immunochemistry

Cell cultures of 3T3 fibroblast cells developed using the NIH Swiss mouse embryo obtained from the American Type Culture Collection (Manassas, VA). 3T3 fibroblasts were cultured in Dulbecco's modified Eagle Medium (DMEM) supplemented with 2 mM glutamine and 10% calf serum. Glass coverslips were coated with poly-L-lysine. When fibroblasts are grown to 70% confluency, cells were split to a subculture routine using 0.25% trypsin/ethylenediamine tetraacetic acid (EDTA) in 37C, in a 5% CO_2 incubator. Functionalized MWCNT and CNF were first autoclaved and then were dispersed in sterilized PBS using an ultrasonicator for 1 h. The concentration of MWCNT and CNF in PBS was 100 μg/mL. 1 mL of this suspension was placed on a coverslip of 25 mm diameter in a 6-well plate for one day. After settling and adhering nanotubes on the coverslips, we replaced PBS with 3T3 cell-containing medium and cultured this for 24 h in a 37 °C, 5% CO_2/95% air humidified cell culture incubator. Fibroblast cells were seeded at a density of 3.5×10^5 cells/mL. Thus the number of cells per surface area was 700 cells/mm^2. There was one control group (cells

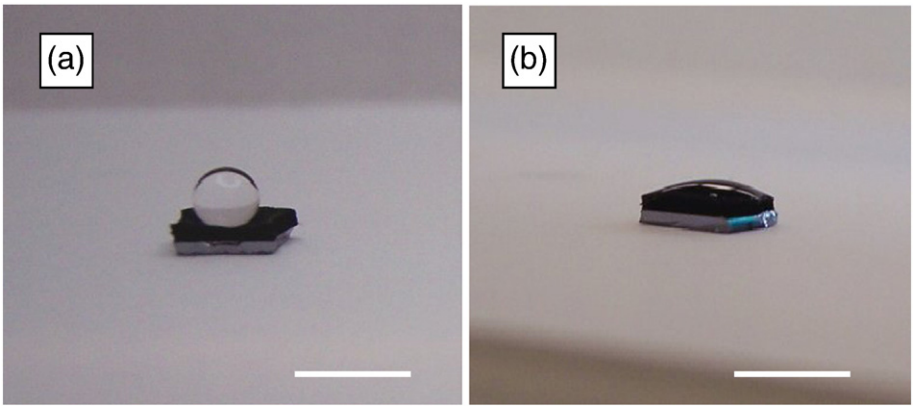

Fig. 3. Images of aligned multi-wall carbon nanotube carpets: (a) photo of a water droplet on a 1 mm long nanotube as grown array, and (b) array after functionalization using acid showing wetting of the surface. Bar is 1 cm.

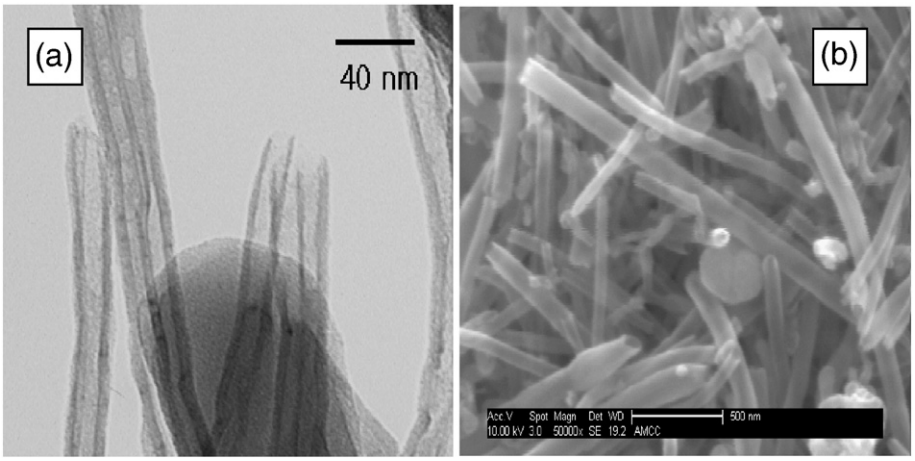

Fig. 4. Effect of functionalization: (a) TEM image of functionalized MWCNT; and (b) ESEM image of functionalized CNF.

grown on glass coverslips) and two experimental groups (cells grown on functionalized MWCNTs and CNFs) used for immunochemistry, the excess medium was blotted from the coverslip, rinsed in PBS, and placed on a coverslip in a chilled coupling jar containing methanol (fixation) that was stored on molecular sieves at −15 °C for 4 min. The final coverslip was incubated in 5% donkey serum in PBS for 10 min (permeabilization). After pipetting off the donkey serum, a primary antibody, a mix of alpha/beta tubulin monoclonal antibodies (alpha, 1:2000, beta, 1:200) purchased from Abcam (ab28439) and BD Pharmingen (55631) was added and incubated for 40 min. After rinsing in PBS 3 times, a secondary antibody, Alexa 488 donkey mouse, is added and incubated for 30 min in the dark. The samples are rinsed three times again and mounted using one drop of mounting medium (Fluoromount-G) on a slide. An Orca-ER Camera/Zeiss Upright Microscope System which is an upright microscope with infinity-corrected optics is used to observe the sample. The scope is motorized and is equipped for epifluorescence, brightfield, DIC, and phase contrast microscopy. An FITC filter cube is used to see α, β tubulins of the specimen using an 100× oil objective. A DIC image is also acquired with the same magnification.

2.4. Cytotoxicity assay

For the MTT assay, 3T3 cells were seeded in 96-well plates at a density of 1×10^5 cells/mL in a 100 μL medium. After 24 h of cell attachment, the plates were washed with PBS and treated with increasing concentrations of each nanotube containing medium for 24 h. Four replicated wells were used for each control. Following 24 h of exposure, the control medium was removed and the cells were rinsed with PBS and 100 μL/well of fresh medium without PBS. 10 μL of MTT (5 mg/mL) prepared in PBS was added to each well and incubated for 2 h. Then the medium was discarded and replaced with PBS followed by 100 μL of DMSO for the removal of dye. The plate was shaken at 250 rpm for 10 min and absorbance was measured at 570 nm.

For Trypan Blue staining, 3T3 cells were seeded in 96-well plates at a density of 1×10^5 cells/mL in a 100 μL medium. After 24 h of cell attachment, the plates were washed with PBS and incubated with 0.1% diluted Trypan Blue in PBS for 5 min. After washing with PBS, the visualization of 3T3 cells with nanotubes was performed under a microscope.

3. Experimental results

Fig. 5 shows the DIC image and stained microtubule fluorescent image of mouse 3T3 fibroblasts with CNFs and MWCNTs. Microtubules of 3T3 fibroblasts were stained clearly using FITC. Since MWCNTs have diameters of approximately 20 nm and lengths of 100 μm–1 mm, bundles or aggregated nanotubes were observed under visual light microscopy. Long aligned functionalized-MWCNTs with high aspect ratio provided good dispersion. Attachment, spreading, and growth of Swiss 3T3 fibroblasts on the surface of functionalized-MWCNTs coated

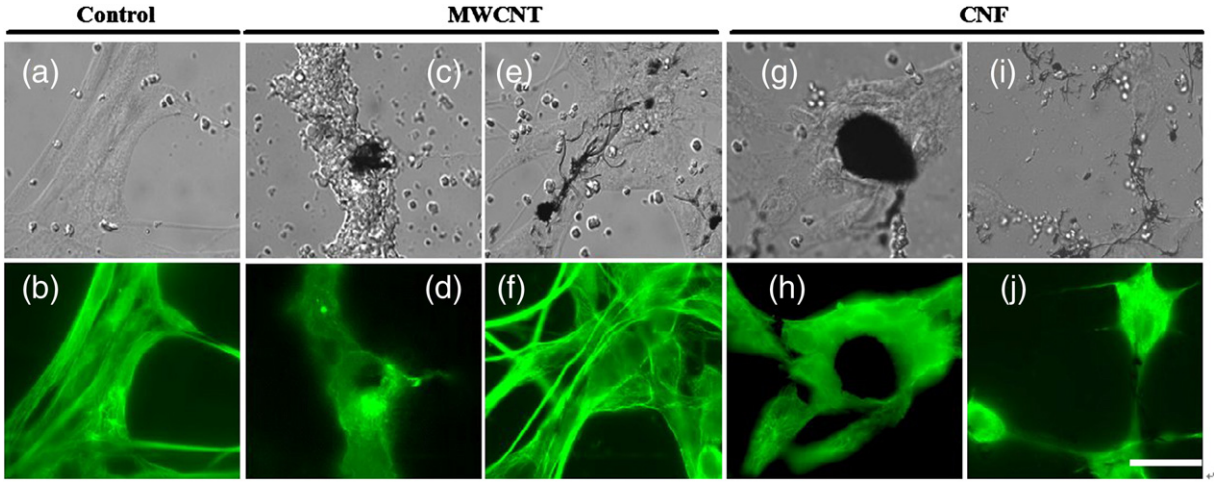

Fig. 5. Fibroblast cell culture on the glass coverslip: (a) DIC image; and (b) stained microtubule fluorescent image: functionalized MWCNT-glass coverslip: (c, e) DIC image and (d, f) stained microtubule fluorescent image: functionalized CNF-glass coverslip: (g, i) DIC image and (h, j) stained microtubule fluorescent image. Bar is 20 μm.

glass slips are shown in Fig. 5. Fig. 5(c–d) shows one large aggregated bundle of MWCNTs surrounded by fibroblasts. Fig. 5(e–f) shows better dispersion of functionalized-MWCNTs on glass cover slips and that mouse 3T3 fibroblasts spread more uniformly on the functionalized-MWCNTs. Adhesion and spreading of tissue cells are related to the substratum surface free energy [9]. While hydrophobic surfaces caused poor spreading, hydrophilic surfaces led to good spreading. Hui Hu et al. [7] investigated another parameter, surface charge, for neuronal cell adhesion. Probably, carboxylic groups located at defects and at the ends of MWCNTs with a high surface area provide high adhesion of fibroblasts. Also, by controlling the density of the MWCNTs on the substrates, the adhesion strength of fibroblasts can be controlled. Since cell adhesion to substrates controls the behavior of cells such as cell morphology, migration, growth, apoptosis, and differentiation [10], functionalized-MWCNT based 3D scaffolds might be a reasonable approach for a cell scaffold. But in order to understand cell interaction with nanotubes, ECM behavior, such as Laminin, FAK, cadherin, fibronection, and collagens, should be studied in the future.

Compared to MWCNTs, CNFs have diameters of approximately 100 nm and lengths of 50–100 μm. Fig. 5(g) shows one bundle of functionalized CNFs surrounded by mouse 3T3 fibroblasts. Fig. 5(g–j) shows how functionalized CNFs interact with fibroblasts. 3T3 fibroblasts adhered to CNF along with functionalized-CNFs. This adhesion ability to fibroblasts is also useful to pattern cells on the substrates [11,12]. Furthermore, Trypan Blue staining was used to show whether 3T3 cells are alive or not, as shown in Figs. 6 and 7. Fig. 6 shows the 3T3 fibroblast cells with different concentrations of MWCNTs from 5 to 50 μg/mL. Trypan Blue stained results show that there were not many dead cells (blue arrows indicate dead cells). DIC and Trypan Blue staining image results of 3T3 cells cultured with different concentrations of CNFs are shown in Fig. 7. As expected, there were not many dead cells for the 24 hour incubation of nanotubes with 3T3 cells. The MTT assay was preformed to study cytotoxicity quantitatively with different concentrations of nanotubes. Fig. 8 shows the MTT assay results of 3T3 fibroblast cells with MWCNTs and CNFs after 24 h incubation. Cytotoxicity was expressed as a mean percentage decrease relative to the unexposed control. The data show a 20% inhibitory effect of 3T3 cell growth at the highest concentration of nanotubes. Thus, short term culture study results from both the Trypan Blue stain and MTT assay show that nanotubes did not significantly alter cell viability. Furthermore, MWCNTs and CNFs enhanced the adhesion of 3T3 cells with low toxicity. However, the long term effect of carbon nanotubes should be explored in the future since the cytotoxicity of fullerenes keeps being reported in the literature [13].

Potentially harmful effects of carbon nanomaterials might come from: (i) the nature of the carbon nanomaterials themselves (i.e. chemical reactivity), (ii) the characteristics of the products made with nanomaterials, and (iii) aspects of the manufacturing processes especially in the strong acid treatment. Proper functionalization and using the bundle form (no dispersed short nanotubes) of nanomaterials did not create any noticeable harmful effects to fibroblasts.

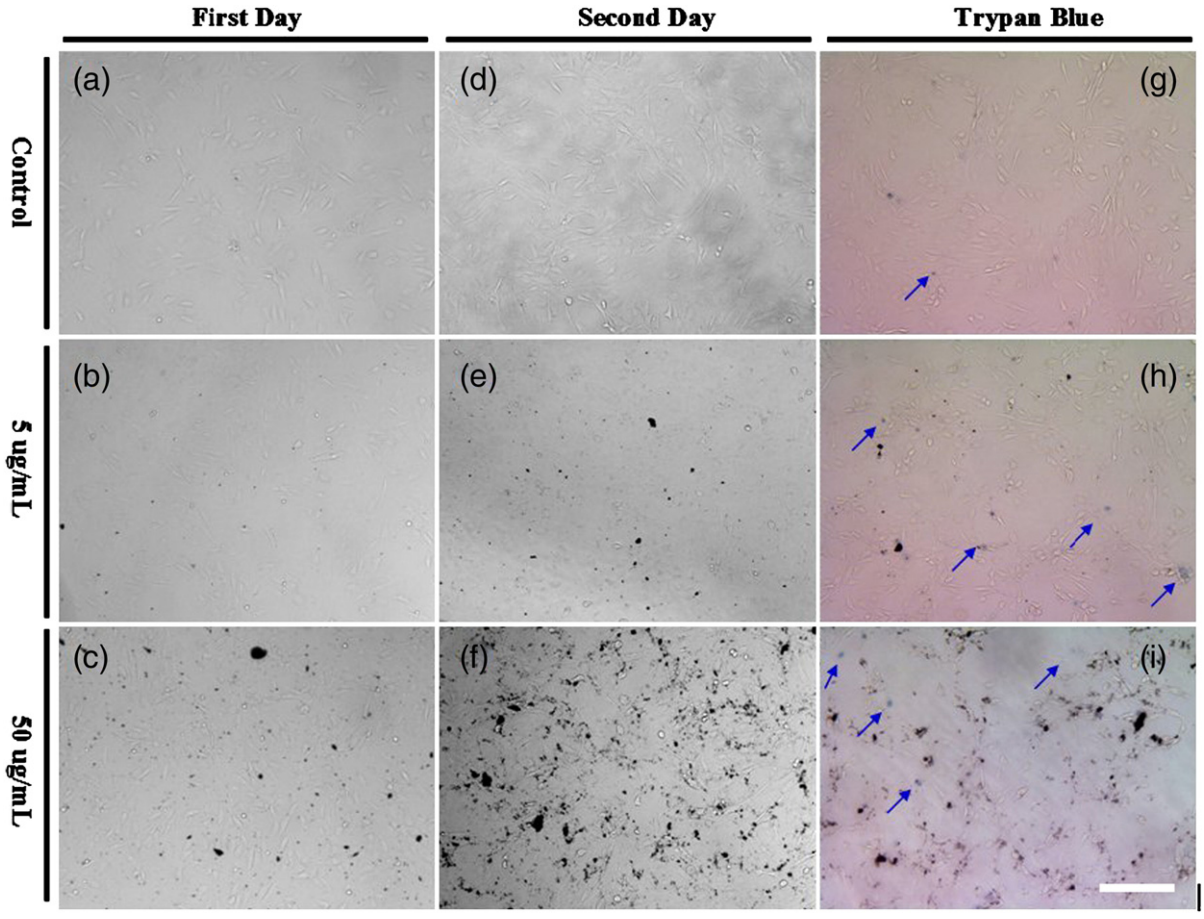

Fig. 6. Fibroblast cell culture on a 196-well plate with different concentrations of MWCNTs in suspension: (a–c) DIC image of fibroblast cell on the first day with (a) control, (b) 5 μg/mL, and (c) 50 μg/mL of MWCNTs and (d–f) DIC image of fibroblast cells on the second day with (d) control, (e) 5 μg/mL, and (f) 50 μg/mL of MWCNTs. Trypan Blue stained image of fibroblast 3T3 cell after 24 h incubation with: (a) control, (b) 5 μg/mL, and (c) 50 μg/mL of MWCNTs. Blue arrows indicate dead cells. Bar is 250 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

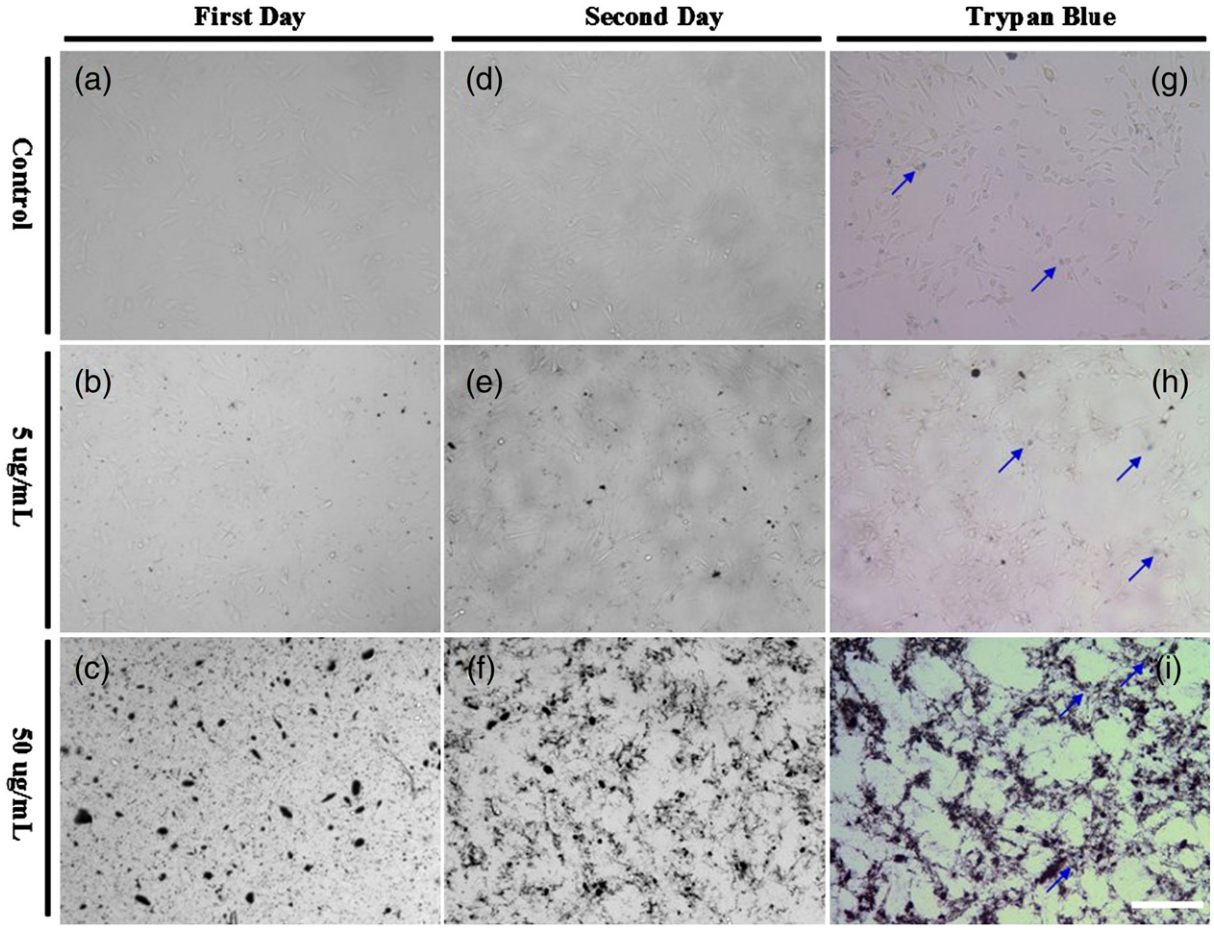

Fig. 7. Fibroblast cell culture on a 196-well plate with different concentrations of CNFs in suspension: (a–c) DIC image of fibroblast cells on the first day with (a) control, (b) 5 μg/mL, and (c) 50 μg/mL of MWCNTs and (d–f) DIC image of fibroblast cells on the second day with (d) control, (e) 5 μg/mL, and (f) 50 μg/mL of MWCNTs. Trypan Blue stained image of fibroblast 3T3 cells after 24 h incubation with: (a) control, (b) 5 μg/mL, and (c) 50 μg/mL of MWCNTs. Blue arrows indicate dead cells. Bar is 250 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Furthermore, the high surface area of the functionalized nanomaterials provided an ideal environment to adhere and spread fibroblasts. These results have considerable potential for scaffold engineering which can be used for biocompatible matrices development for tissue substitutes. In particular, excellent mechanical properties, high flexibility, and a surface with nanoscale features provide the ideal solution for restoring, maintaining, or reinforcing damaged tissue. A. Monteiro-Riviere et al. reported that unmodified MWCNTs were present within cytoplasmic vacuoles of the HEK and induced the release of the proinflammatory cytokine interleukin 8. This paper indicates that a careful study of cytotoxicity is needed for future tissue graft development.

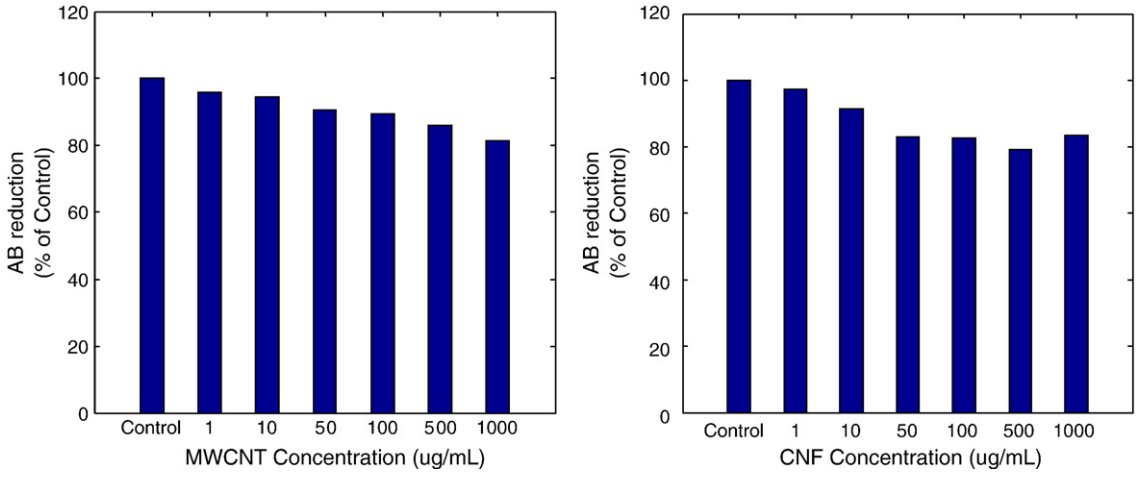

Fig. 8. MTT assay of 3T3 fibroblast cells with (a) MWCNTs and (b) CNFs after 24 h incubation. Data were averaged and expressed as percent of control.

4. Conclusions

Fibroblast cell behavior on the surface of functionalized nanomaterials was successfully monitored. Strong acid treatment made the nanomaterials more hydrophilic which increased the adhesion of the fibroblast cells. Strong adhesion of the 3T3 fibroblast cells to the functionalized nanomaterials was observed. There was no significant difference observed between fibroblast cell adhesion for MWCNT and CNF. The results of this study indicate that nanomaterials might be used as scaffolds for tissue. Further work is needed to understand why nanoscale materials promote tissue growth and to study long term biocompatibility of the nanomaterials.

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