A Preliminary Study on Using Multi-Nozzle Polymer Deposition System to Fabricate Composite Alginate/Carbon Nanotube Tissue Scaffolds

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Abstract

Three-dimensional composite alginate/single wall carbon nanotube (SWCNT) scaffolds encapsulated with endothelial cells were fabricated by a multi-nozzle biopolymer freeform deposition system. This system enables the converting of CAD designed scaffold pattern into process toolpaths and the use of computer control program to guide the nozzle deposition at spatial position for layered fabrication of 3D tissue scaffolds. The morphological, mechanical, structural and biological properties of as-fabricated scaffolds were characterized by optical microscope, SEM, Microtensile testing machine, Alamar Blue Assay, and Live-Dead Assay, respectively. The multi-nozzle deposition system demonstrated a highly efficient and effective process to build tissue scaffold or cell embedded constructs. Characterization results showed that the incorporation of SWCNT into alginate not only enhanced the mechanical strength of the scaffolds but also improved the cell affinity and the interaction with substrate. Further cell culture experimental results also showed that the incorporation of SWCNT in alginate enhanced endothelial cell proliferation compared with pure alginate scaffold.

1. Introduction

Tissue engineering is an interdisciplinary field which applies the principles of engineering and the life sciences toward the development of biological substitutes that restore, maintain, or improve tissue function [1]. In the tissue regeneration process, scaffold may play important role for cell attachment, proliferation and new tissue regeneration [2-5]. An ideal tissue scaffold is often required to have certain degree of mechanical strength and stiffness, nutrient transport and diffusion properties, and suitable surface chemistry in order to produce a desirable cellular behavior. Composite scaffolds which consist of two or more biocompatible materials sometimes can be better used as tissue scaffolding materials. This paper summarizes some preliminary results of using single-walled carbon nanotube as a suitable reinforcement for freeform-fabricated composite alginate/SWCNT tissue scaffold. The primary objectives of this research are as follows: 1) explore the feasibility of biopolymer deposition for solid freeform fabrication of SWCNT enhanced 3D alginate hydrogel composite tissue scaffolds; 2) study the morphological, structural and mechanical properties of the freeform fabricated SWCNT/alginate structures; and 3) Investigate the cell viability and cell proliferation ability of SWCNT enhanced structures and their applications in tissue engineering.

2. Materials and Methods

2.1 Materials

High-purity single-walled carbon nanotubes (SWCNT) with average diameter of 1.5 nm were obtained from Rice University according to established high pressure carbonmonoxide (HiPco)

technique [6]**.** The SWCNTs were purified to reach a purity of up to 97 mol % [7]. Single-walled carbon nanotubes solution was prepared by dispersing powder form single-walled carbon nanotubes in sterile DI water and then by sonicating at 55^{o}C for 3 hours to obtain homogenous suspension. Then this suspension was sterilized by Napco^{TM} Autoclave (VA, USA) for 20 minutes at 80^{0}C.

The alginate with a 61% mannuronic acid content was purchased from Sigma-Aldrich. A 1.5% (w/v) solution of sodium alginate is prepared by suspending the powdered alginate in deionized (DI) water. The suspension was stirred for 2 days at room temperature. In order to sterilize the alginate solution a filtration process was used. Solution was filtered through a series of membrane filters of pore sizes 1.2 μm, 0.8 μm, 0.45 μm, and 0.22 μm. This process removes some contaminants such as proteins and polyhenols [8].

The aqueous solution of composite alginate- SWNT was prepared by mixing alginate and SWCNT together in a bottle under aseptic environment. Then the mixture was stirred at room temperature for overnight to get even distribution of SWCNT in the solution. In the final composite solution, alginate concentration was 1.5% (w/v) and the concentration of SWCNT relative to alginate in the final solution is 1% (w/w).

2.2 Cell-composite material suspension

Rat adrenal medullary endothelial (CAME) cells (blood vessel-forming cells) were obtained from the American Type Culture Collection (Arlington, VA) and were used at passage numbers 22-27 in the experiments. Cells were cultured with Dulbecco's Modification of Eagle's Medium (DMEM) consisting of 9% fetal bovine serum (FBS; Hyclone), 50 I.U./mL penicillin (Cellgro), 50 μg/ml streptomycin (Cellgro), 0.125 μg/ml amphotericin-B (Cellgro) and 2mM of L-glutamine (Cellgro,VA) in incubator conditions at 37°C with 5% CO2 and 95% air environment. Medium was refreshed every 2-3 days. When the cells become confluent, they were unattached from the flasks and counted by hemocytometer (Hausser Scientific Company). After counting the cells were mixed (encapsulated) with alginate-SWCNT composite material with a seeding density of 1.0×10^{6} cells/mL.

2.3 Scaffold design and fabrication

The geometry of the scaffolds was first designed with 0/90/0° lay-down pattern in CAD model and then converted into STL format. The STL file was then loaded into the process software to define the toolpaths for freeform deposition of the composite alginate/SWCNT solution. A proprietarily developed biopolymer deposition system was used in this study (Figure 1). This system is capable of extruding biopolymer solutions and living cells for freeform construction of three-dimensional tissue scaffolds [9]. During the fabrication process, an in-situ dual nozzle cross-linking method was used. This method deposits the aseptic alginate/SWNT solution and the calcium chloride solution simultaneously from two nozzles (Figure 2). When the solutions from the two nozzles were extruded and got contact, the alginate anion and the calcium cation were crosslinked, thus the hydrogel was formed. By moving the nozzle tip over a substrate in the designed path, the material was laid down in the form of line structures to create the desired model. This process can be repeated layer by layer to develop a freeform fabricated part (Figure 2). The air pressure of the pneumatic valve in this study was 8.0 psi. The nozzle inner

diameter and the nozzle traveling speed were 330 μm and 10 mm/s, respectively. The cross-linking time for each layer was 6 seconds.

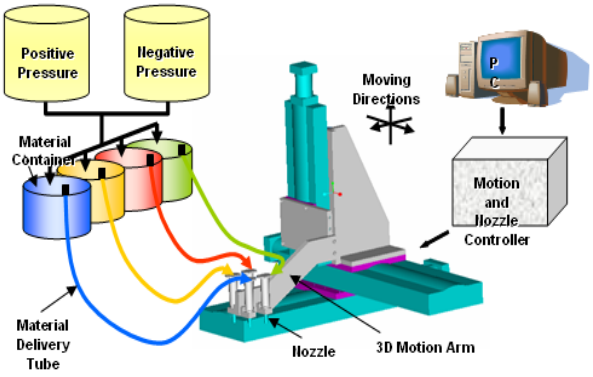


Figure 2: Schematic diagram of multi-nozzle deposition system

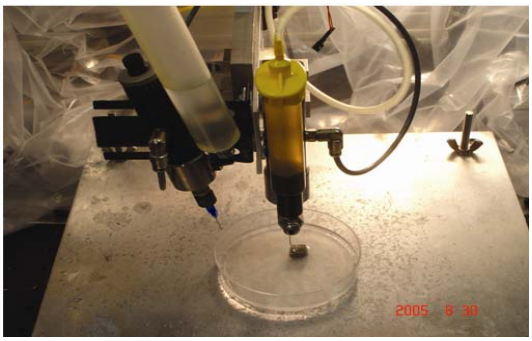
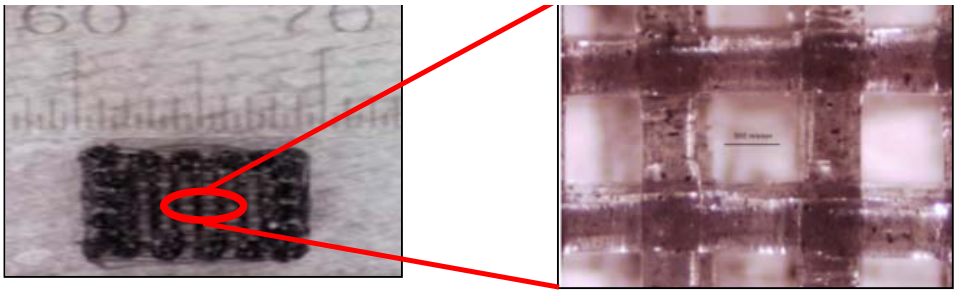


Figure 2: Schematic of in-situ dual nozzle cross-linking

Two groups of scaffolds were studied. In the first group, alginate with 1% w/w SWCNT-cell mixture was used as the scaffolding materials and a pure alginate-cell mixture was used as the control group. All scaffolds used in this study have same design parameters with 10mm (length) ×10mm (width)×5mm (height). Each scaffold consists of 10 layers with 300 μm strut width and 450 μm pore size. The overall scaffold porosity is 70%. The morphologies of first and second group of scaffolds are given in Figure 3 and Figure 4, respectively.

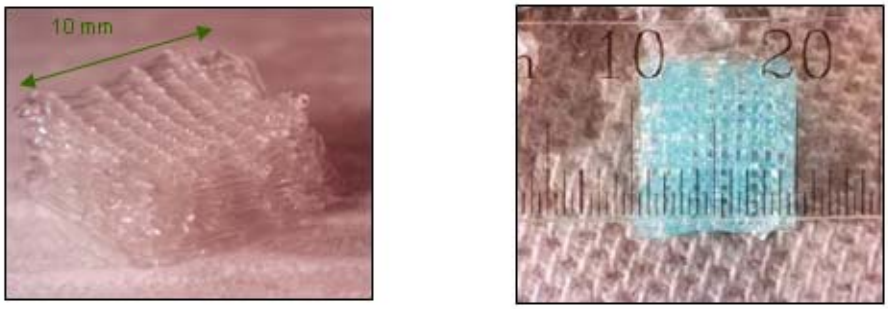


(a) (b)

Figure 3: a) Top view of scaffold made with alginate-1% w/w SWCNT-cell mixture
b) Scaffold composite alginate/SWCNT-cell mixture

During the fabrication process the system parameters were kept the same for all scaffolds. The following process parameters were used: the air pressure of the pneumatic valve was 8.0 psi, the nozzle inner diameter 330 μm, the nozzle moving speed 10 mm/s, respectively, and the cross-linking concentration 5% and the cross-linking time for each layer was 6 seconds.

Immediately after fabrication, all scaffolds were washed with DMEM for five times to remove the residual calcium chloride. Then the scaffolds were put in a sterile 24-well plate (Costar, NY) with 1mL fresh medium and placed into the incubator at 37°C with 5% CO_2 and 95% air environment. The medium was changed every 2-3 days.



(a) (b)

Figure 4: a) Isometric view of scaffold made with pure alginate-cell mixture;
b) Top view of scaffold made with pure alginate-cell mixture

3. Characterization of Morphological, Mechanical and Biological Properties

3.1. Spectral Analysis by Raman Spectroscopy

Raman spectroscopy is used to detect the presence of SWCNTs in the fabricated scaffolds and perform comparative study of the structure-property relationship between pure alginate and SWNT containing alginate samples. A Reinshaw Raman micro-spectrometer Ramanscope 1000 is used to record the spectra. A He-Ne laser with 633 nanometres excitation wave length is used. The He-Ne laser corresponds to the equivalent photo energy of 1.91 eV (laser excitation).

3.2 Characterization of mechanical properties of alginate strands

Tensile property was characterized by a Kawabata Evaluation System (KES-G1, Kato Tech Co. Japan) according to routine mechanical testing methods for fabric materials [10]. Both alginate sample without carbon and sample with SWNT were tested for comparison. The samples to be tested were Φ1mm x 12mm slim strips and seven such samples were taken from each scaffold. The sample was mounted onto a rectangular paper frame (Figure 5), leaving a 7mm gauge length for mechanical loading. An extension rate of 0.2 mm/s, sensitivity of 2×1(10V = 40g), and frequency of 50 Hz were used in the tensile tests. Load-deformation data were recorded and the stress-strain curve was constructed.

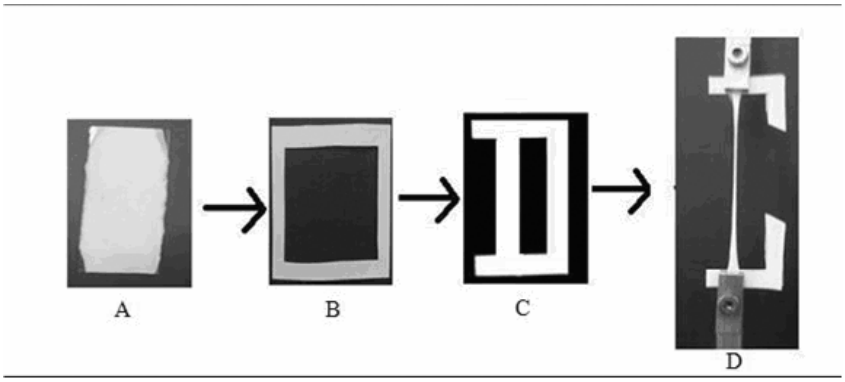


Figure 5: Schematic of mechanical test sample
A. sample; B. rectangular paper frame;
C. sample mounted on paper frame; D. sample undergoing tensile test.

3.3 Cytocompatibility and Cell Interaction

In order to investigate the cell interactions with composite alginate –SWCNT materials, three experiments were conducted. In first experiment, cells were seeded on the top of the bulk composite scaffolds. In the second one, cells were mixed with composite alginate-SWCNT solution and final solution was deposited inside the well plates by pipet. In the third experiment, cells were encapsulated inside the composite alginate-SWCNT solution and final solution was printed by using multi-nozzle deposition system. All experiments were conducted for 14 days and cell viability was evaluated by alamarBlue (aB) assay and Live-Dead assay. The proliferation profile of scaffolds in each experiment was measured by alamarBlue assay on Day 0, Day 3, Day 7, and Day 14 respectively. At the day of measurements, alamarBlue solutions were added to each sample with 10% v/v ratio and allowed to react for 5 hours at incubation condition. Then, 800 μL solution was taken out of each incubated well and put into microplate reader (TECAN, USA) to measure the fluorescence density of the solution.

In order to qualitatively evaluate the cell viability, Live-Dead assay was used for each experiment. Because of the toxic nature of the Live-Dead assay markers, the Live-Dead assay study was done at the last day of the experiments (Day 14). In order to apply Live-Dead cell assay, samples were first washed with PBS solution for several times to remove the medium. Then they were submerged in the solution containing PBS with 8μL ethidium bromide homodimer and 2μL calcein and were incubated for 1 hour at 37°C with 5% condition incubator. After that, the samples were examined in an inverted fluorescent microscope (Leica DM IL) using filters with scan range from 500 nm to 700 nm and from 420 nm to 620nm. Representative images were captured using a digital camera and Spot Insight software (Spot Diagnostic Inc, US).

As a first attempt to look how Rat adrenal medullary endothelial cells (blood vessel-forming cells) would interact with composite alginate–SWCNT materials, Rat adrenal medullary endothelial (RAME) cells were seeded on the top of the scaffolds with a 1×106 cell/mL in both control and composite material groups. In order to make the cells adhere onto the sample surfaces, the seeded samples were incubated at 37°C in a 5% CO_2 condition overnight. After incubation, the samples were removed from the original cultured well plate to a new plate in order to ensure that only the cells that had attached to the materials were taken into account for later characterization. 1mL fresh medium was put in each well and refreshed every 2-3 days. Control group of scaffolds and composite alginate-SWCNT scaffolds were incubated for up to 14 days at 37°C and 5% CO_2 in DMEM. At day 0, day 3, day 7 and day 14 alamarBlue (aB) assay and at day 14 Live-Dead assay were performed.

In order to check the cell viability and interaction inside the composite alginate-SWCNT material, Rat adrenal medullary endothelial (RAME) cells encapsulated with 1×106 cell/mL seeding density inside the composite alginate–SWCNT material. Composite material with cell suspension was deposited into well plates by pipeting and crosslikned with 0.5% $CaCl_2$. After deposition, scaffolds were washed out with DMEM for five times to remove the residual $CaCl_2$. Then, scaffolds were removed into a new well plate and 1mL fresh DMEM was put in each well. Control group (pure alginate) scaffolds and composite alginate-SWCNT group of scaffolds were incubated for up to 14 days at 37°C and 5% CO_2 in DMEM. At day 0, day 3, day 7, and day 14, alamarBlue assay and at day 14 Live-Dead assay were performed.

The last experiment was conducted for evaluating the performance of the multi-nozzle deposition system for printing the cells inside the composite alginate-SWCNT composite material. RAME cells were encapsulated with 1×106 cell/mL seeding density inside the composite alginate–SWCNT material. Then cell suspended composite solution was placed to first pneumatic syringe of the multi nozzle deposition system and for crosslinking 0.5% CaCl2 was put into second pneumatic syringe. System process parameters are kept constant as described above. After the deposition, scaffolds were washed out with DMEM and scaffolds were removed into a new well plate and 1mL fresh DMEM was put in each well. Control group (pure alginate) scaffolds and printed cells inside the composite biomaterial were cultured in DMEM for up to 14 days at incubator conditions. At day 0, day 3, day 7, and day 14, alamarBlue assay and at day 14 Live-Dead assay were performed.

4 Results

4.1. Structural change by Raman Spectroscopy

Raman spectrums were obtained on both alginate sample without SWCNT and composite sample with 1% SWCNT shown in Figure 6. The spectra of the scaffolds containing SWCNT showed very sharp peaks of Raman active mode near 1590 cm^{-1} which are believed to be associated with the G band corresponding to the tangential displacement of the carbon-carbon (C-C) bond stretching motion of graphite in the nanotube walls [11]. The very large intensity of the highest peak indicated a high purity of SWCNT. Several intense peaks of SWCNTs which correspond to the Radial Breathing Mode (RBM) were seen near 210 cm^{-1}. These peaks are typical characteristics in the SWCNT spectrum and were evidences of the successful inclusion of SWCNT in the polymeric scaffolds. The sample without carbon showed no dominating peak for either graphite or SWCNTs, which was expected. Intensity of spectra indicated the amount of nanotubes. Presence of multiple RBM peaks indicated wide range of tube diameter distribution in the scaffold.

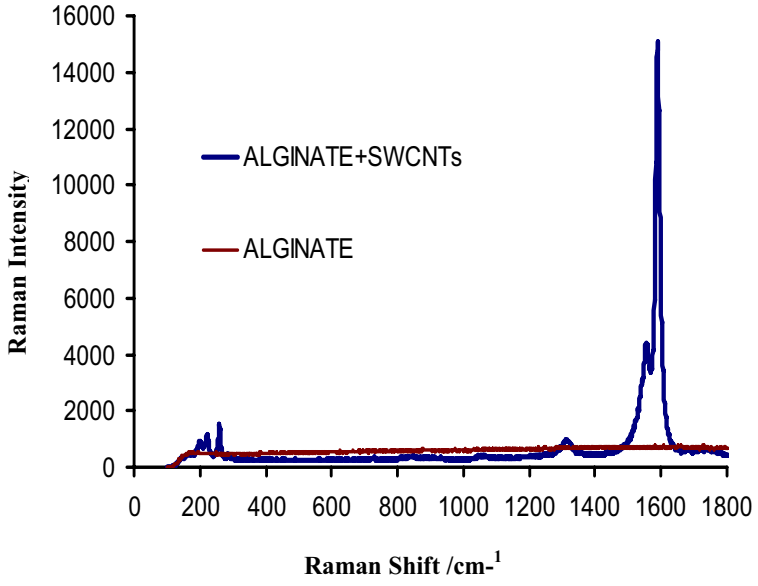


Figure 6: Raman spectrogram of hydrogel scaffolds

The diameter of the carbon nanotubes can be calculated using the peaks in the RBM.[46] The frequency (ω_R) of the RBM is inversely proportional to the nanotube diameter (d) as $\omega_R \sim 224$ cm^{-1} (nm)/d. The peaks in the RBM were at 260, 222 and 198 cm^{-1} and the average nanotube diameter was calculated using all three peaks and found to be 1.00±0.13 nm.

4.2. Mechanical property of individual strands

Samples from each type of scaffold were tested to determine their mechanical properties such as elastic modulus, tensile strength and elongation at break. The stress-strain plot is shown below (Figure 7). From the plot we can determine the modulus (initial slope of the curve), the ultimate stress and the elongation at break. The numerical values for the maximum stress, the modulus and elongation at break for both samples with and without SWNT have been shown below (Table 1).

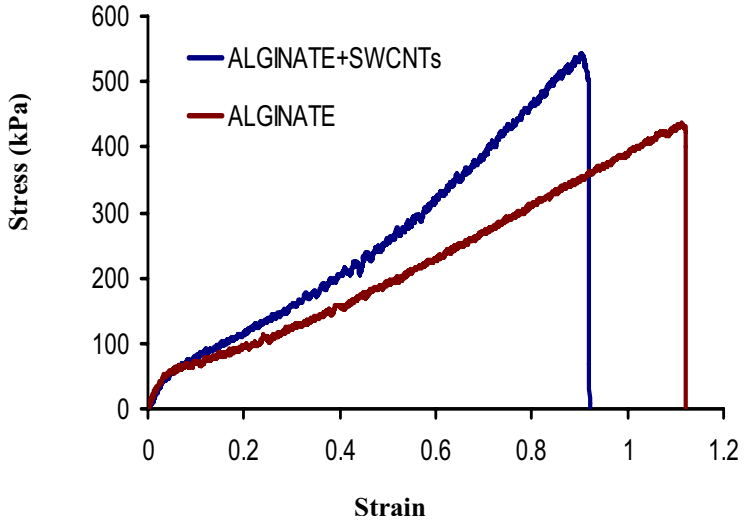


Figure 7: Tensile stress-strain relation of hydrogel strips

Table 1: Mechanical property values for scaffolds.

Sample	Maximum Stress (KPa)	Elastic Modulus (MPa)	Elongation at Break (%)
Pure Alginate	436	1.41	112%
Composite Alginate with 1% CNT	542	1.44	92%

The stress-strain curves of both types of scaffold displayed a nonlinear plot up to the breaking point (Figure 13). We can see that the samples with SWCNTs were able to withstand a higher ultimate stress than the corresponding unreinforced scaffolds were. While in the linear elastic region (< 4%), we didn't see significant difference of their elastic moduli (Table 1). This can probably be explained by the alignment of the SWNT along the strip orientation during the mechanical strengthening stage, thereby improving the tensile strength. On the other hand, the elongation at break of alginate/SWNT sample was smaller than that of pure alginate sample; hence after adding carbon to the alginate, the material became more brittle.

4.3 Cytocompatibility and cell interaction

In current study, fluorescence intensity measurements taken from microplate reader at day 0, day 3, day 7, and day 14 were plotted versus to the incubation period of the experiment. In all experiments, fluorescence intensity data were normalized according to the Day 0 fluorescence intensity. Results presented in Figures 8 and 9 were obtained from pipetted deposition and Results in Figure 11 were obtained by using multi-nozzle deposition system.

From Figure 8, we observed that on the alginate-SWNT composite scaffold surfaces the RAME cells were continuously proliferated. In the presence of SWCNT in alginate, the number of RAME cells on composite material scaffolds is increasing and exceeded more than 4 times at the day 14 than the number at day 0. The cell proliferation rate of the alginate-SWNT composite sample was much higher than that of the pure alginate. This demonstrated the biocompatibility of the single walled carbon nanotube in the alginate scaffold to RAME cells. The trend of the cell proliferation also reveals that the presence of single walled carbon nanotube in alginate scaffold is likely to enhance the RAME cell growth behavior.

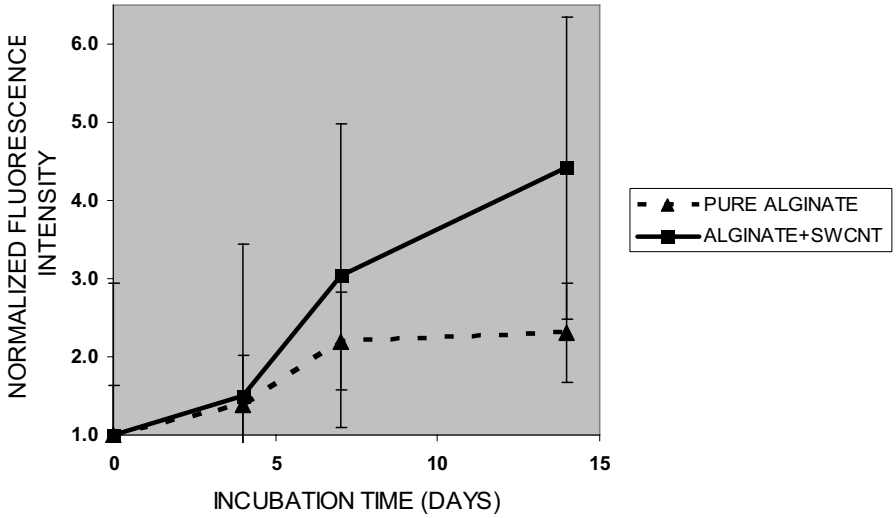


Figure 8: Comparison of the proliferation of seeded RAME cell on the top of the composite alginate-SWCNT scaffolds and top of pure alginate during the 14 days incubation

Figure 9 compares the cell proliferation rate of encapsulated RAME cells inside pure alginate and the alginate-1%(w/w) SWCNT composite scaffold. It is interesting to see that that number of cell inside the composite scaffold increases at a constant rate within the first 7 days incubation, and then reaches to a saturation stage, while the number of cells inside the pure alginate decreases in the first 3 days incubation and then reaches to a saturation stage. This again shows an enhanced cell proliferation property for composite alginate-SWCNT scaffold compared to pure alginate scaffold.

From both Figure 8 and Figure 9, we all observed that the proliferation rate of RAME cells increase in the encapsulated composite alginate-SWCNT scaffolds or seeding on the top of the composite alginate-SWCNT scaffolds. In either case, the cell proliferation property was enhanced in the presence of SWCNT in alginate. Figure 10 suggest that there are more cells inside the composite alginate-SWCNT. This can be further seen from the Alamar Blue assay

results (Figure 10). From Live-Dead assay it can also be seen that inside the alginate-SWNT composite sample there are more live cells aggregated at the substrate where SWCNTs appear, or to be aggregated with SWCNTs.

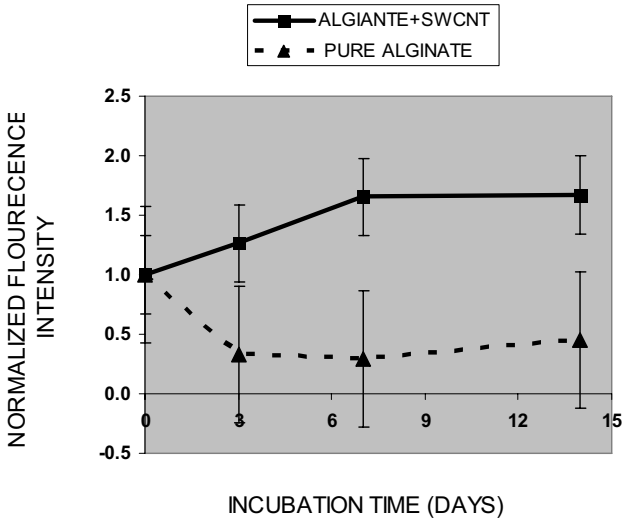
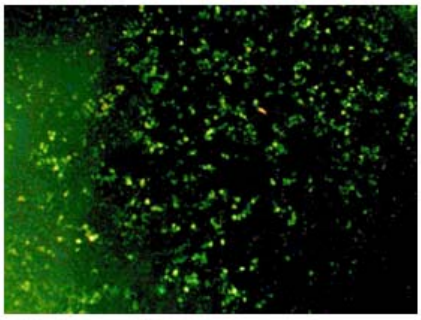
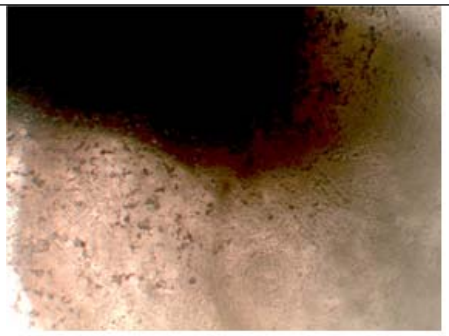


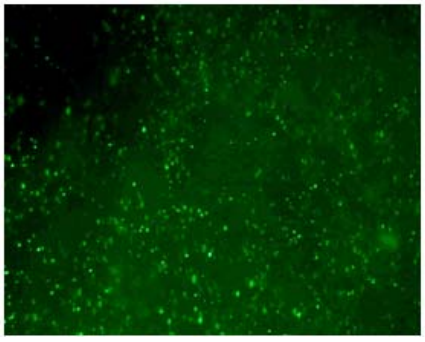
Figure 9: Comparison of the proliferation of encapsulated RAME cell within the composite alginate-SWCNT scaffolds and within pure alginate during the 14 days incubation



a) Fluorescent image of live cells inside the alginate-SWCNT composite material



d) Optical image of live cells inside the alginate-SWCNT composite material



c) Fluorescent image of live cells inside the pure alginate

b) Optical Image of cells inside the pure alginate

Figure 10: Representative images of inverted fluorescent microscope of RAME cell inside the composite alginate-SWCNT and inside pure alginate at day 14

Figure 11 shows the RAME cell proliferation inside the control scaffold and composite scaffold deposited using the multi-nozzle deposition system (MNDS). Result of Alamar Blue assay was given in Figure 10 for 14 days. Cells encapsulated inside the composite alginate-1%(w/w) SWCNT again show a better proliferate property compared to cells encapsulated inside the pure alginate scaffold. A comparison of the fluorescence intensity for two groups of scaffolds deposited via pipeting or multi nozzle deposition system is given in Table 2.

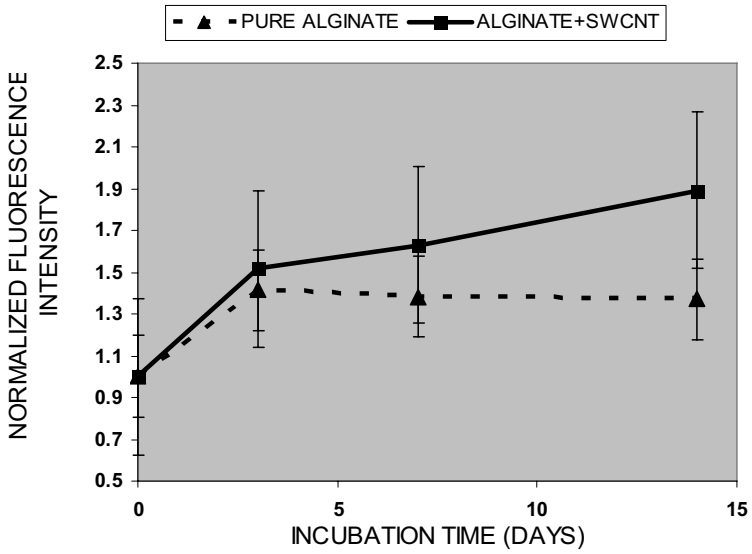


Figure 11: Number of RAME cell increase inside the pure alginate and composite alginate-SWCNT scaffolds deposited using the multi-nozzle deposition system

Table 2: Comparison of fluorescence intensity of both pure alginate and composite material deposited via pipeting or multi nozzle deposition system

	FLUORESENCE INTENSITY	
	Pure Alginate	Alginate with 1%(w/w) SWCNT
Deposited by Pipet	53022	13912
Deposited by MNDS	14553	14043

4. Discussions

In this study, a feasibility of single-walled carbon nanotube as a suitable reinforcement for freeform-fabricated three-dimensional alginate tissue scaffold was studied. The Raman spectra of the scaffolds provided strong evidence of the presence of high-purity SWNT in the fabricated scaffolds as the spectra was identical to the known spectra of SWCNTs. Essentially, sufficient mixing of the polymer and SWCNT resulted in the incorporation of the SWCNT within the polymeric hydrogel. The mechanical tests showed that the incorporation of SWCNT in the polymeric fibrils would improve the scaffold mechanical properties. There was an increase in ultimate strength of the reinforced scaffolds as compared to the unreinforced ones. Evaluated by seeding the scaffold with RAME cells, the Alamar-Blue assay showed that the SWCCNT incorporated structure would aid in cell adhesion and proliferation. The Live cell assay revealed the presence of much more viable cells on the scaffolds with SWCNT at the end of 14 days. We believe that the enhanced cell growth behavior can be contributed by the increased number of

atoms and electron delocalization within the scaffold or on the surface due to the presence of SWCNT. This can alter surface energetic of scaffold and results in an improved interactions of proteins within the cell substrate that trigger subsequent cellular adhesion [12-14]. The reason of this result may be the nanotopographic properties of SWCNT in terms of having high surface to volume ratio which leads to better attachment for RAME cells.

It is important to note that the single-walled carbon nanotubes utilized in this study were not functionalized with bioactive molecules; enhanced endothelial cell function was demonstrated on unmodified SWCNTs. Therefore, future work will focus on the effect of fictionalization of SWNT on the reinforcement of scaffold mechanical and biological properties. We also would like to see how these property reinforcements will change with different SWCNT concentrations. Moreover, in order to assess the actual behavior of the scaffold at the implant site, in vivo study needs to be carried out.

Depositing live cells via pipeting cells inside the pure alginate shows generally higher fluorescence intensity than depositing via MNDS, as shown in Table 2. We believe that there is s process induced cell damage during the multi-nozzle deposition process because the pipette pressure is lower than the pressure used in the MNDS process. We further speculate that SWCNT provides some kind of lubricating effect which can alleviate the friction force inside the nozzle between cells-cells and cell-alginate during the deposition process, thus reduce the cell damage. More rigorous study will be conducted in the future to understand the process induce cell damage for MNDS process.

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