Biocompatibility of Carbon Nanotubes for Cartilage Tissue Engineering

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ABSTRACT

Carbon nanotubes (CNT) have the potential to become an important component of scaffolding in tissue engineering, due to their unique physical properties. However, one major limitation of CNTs that must be overcome is their inherent cytotoxicity. In this study, we assessed the long-term biocompatibility of CNTs for chondrocyte growth. The effect of CNTs on chondrocyte viability and biochemical deposition has been examined in two dimensional (2D) cultures, and in three dimensional (3D) composite materials consisting of hydrogels and CNTs. The exposure of chondrocytes to CNTs was carried out up to 14 days in 2D culture and 21 days in 3D agarose composites. Our results suggest that functionalized CNTs alter the viability and metabolic response of cells over the 2 to 3 week duration. Interestingly, the results of this study also suggest the dose dependent effect of CNTs on cellular responses vary between 2D and 3D cultures, suggesting that chondrocytes tolerate the presence of CNTs at greater concentrations in 3D more than in 2D cultures. In addition, CNTs appear to have a stimulatory metabolic effect on chondrocytes in 3D cultures, reflected by enhanced production of glycosaminoglycans (GAGs) and collagen deposition. These findings support the notion that optimization of the use of nanotubes in cell-based therapies should be performed in 3D systems directly, and that CNTs appear to promote cellular growth and metabolic activity of chondrocytes.

Keywords: Carbon Nanotubes, Cytotoxicity, Tissue Engineering, Biocompatibility, Cartilage.

1 INTRODUCTION

Carbon nanotubes (CNTs) are cylindrical allotropes of carbon that are nanometers in diameter and posses unique physical properties, positioning them as ideal materials for studying physiology at a single cell level. CNTs have the potential to become a very important component of medical therapeutics, such as in (a) drug delivery system [1], (b) existing as an interfacial layer in surgical implants [2,3], or (c) acting as scaffolding in tissue engineering [4,8]. While some studies have explored the use of CNTs as a novel material in regenerative medicine, they have not yet been fully evaluated in cellular systems. One major limitation of CNTs that must be overcome is their inherent cytotoxicity. The goal of this study is to assess the long-term biocompatibility of CNTs for chondrocyte growth. We hypothesize that CNT-based material in tissue engineering can provide an improved molecular sized substrate for stimulation of cellular growth, and structural reinforcement of the scaffold mechanical properties. Here we present data on the effects of CNTs on chondrocyte viability and biochemical deposition examined in composite materials of hydrogels + CNTs mixtures that supports our hypothesis. Also, the effects of CNTs surface functionalization with polyethlyne glycol (PEG) or carboxyl groups (COOH) were examined.

2 METHODS

Purified single wall carbon nanotubes (SWNTs) functionalized with terminal carboxylic acid (SWNT-COOH) or covalent polyethylene glycol (SWNT-PEG) (Carbon Solutions Inc., Riverside, CA) were UV-sterilized, suspended in DI water, and sonicated for ~3 hours to arrive at a stable, homogenous suspension at room temperature (RT). Chondrocytes were isolated from immature bovine articular cartilage (1-3 days old) using enzymatic digestion, as previously described [5].

2.1 Effect of CNTs on cell viability (2D Culture)

Chondrocytes were plated at 1 x 10^{6} cells/cm^2 and cultured in high glucose DMEM + 10% FBS + 1X penicillin for up to 14 days. Cells were exposed to media containing one of the following treatment groups: a) Control, (b) Sonicated Media (SM), (c) 0.1 mg/ml SWNT+COOH, (d) 0.01 mg/ml SWNT+COOH, (e) 0.1 mg/ml SWNT+PEG, (f) 0.01 mg/ml SWNT+PEG. Chondrocyte viability was measured with a fluorescence microplate assay, using a Live-Dead assay kit (Molecular Probes, Portland, OR). Cell viability was reported as % live cells, on day 3 (D3), 7 (D7) and 14 (D14). Absolute cell percentages were acquired by killing all cells in 1 well per group using 0.25% digitonin. Fluorescence microscopy images of the cells were also taken using an inverted scope.

2.2 CNT-Hydrogel Constructs (3D Culture)

Chondrocytes were seeded at a final density of 10 x 10^6 cells/ml in composite mixtures of 2% low melt agarose ± SWNTs. Agarose/SWNT mixtures were sonicated prior to addition of cells, and final mixtures were cast between 2 glass plates. Cylindrical constructs were prepared (∅5mm/~1.7mm thick) from 4 treatment groups: (a) Control, (b) 0.1 mg/ml SWNT+PEG, (c) 1.0 mg/ml SWNT+PEG, (d) 0.1 mg/ml SWNT+COOH. Disks were cultured up to 21 days in high glucose DMEM supplemented with 10 ng/ml TGF-β3, 1% ITS, 100nM dexamethasone, 50μg/ml L-Proline, 100 μg/ml sodium pyruvate, and antibiotics (penicillin, streptomycin).

Water content of the disks was measured by lypholization. In addition, the biochemical content of molecules typically synthesized by chondrocytes was measured, as an indication of biological synthesis. Glycosaminoglycans (GAG) are extracellular matrix molecules typically found in cartilage, and are indicative of the presence of differentiated chondrocytes in culture. The GAG content in these constructs was assessed using a calorimetric assay (Blyscan Assay; Biocolor, U.K.). Double stranded DNA content in the samples was also measured using Quant-IT assay (Molecular Probes), against calf thymus DNA standards. Cellular composition was also assessed histologically on paraffin embedded sections to determine glyocoaminoglycan (GAG), (Alcian blue) and collagen deposition (picrosirius red) differences among the various culture groups. Cellular viability in the constructs was also assessed using the Live/Dead viability assay, and imaged on an inverted fluorescence microscope.

At each time point, the biomechanical properties of constructs were measured under unconfined compression using an Instron testing frame (equipped with 50N load cell), similar to previously described protocols [5]. Three stress relaxation compressions were applied (5% strain per ramp), and the Young's modulus (E_Y) was determined as the slope of the stress-strain curve.

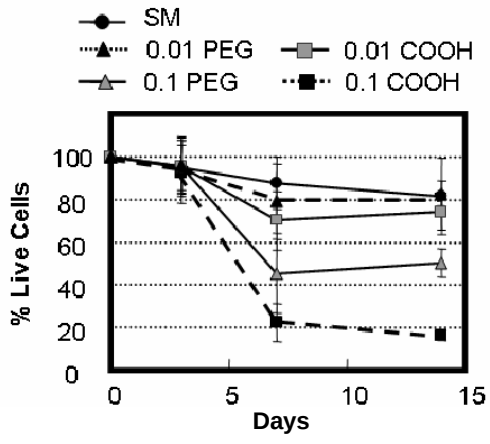

Figure 1: Effect of SWNTs on chondrocyte viability in 2D culture over 2 week period.

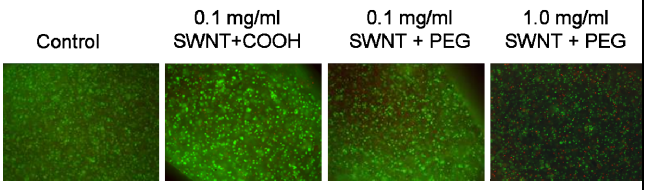

Figure 2: Viability of chondrocytes at Day 7 in varying CNT hybrid constructs. Green indicates live cells, red indicates dead cells.

3 RESULTS

3.1 Effect of SWNTs on cell viability (2D Culture)

Chondrocytes maintained comparable viability in all groups, up to Day 3 in culture (Figure 1). Cells cultured in sonicated media (SM) exhibited limited loss in viability by Day 14 (82 ± 18% live). The addition of CNTs at 0.01 mg/ml resulted in comparable viability for SWNT+PEG (83±6%) and SWNT+COOH (76±9%). However by Day 7, the percentage of live cells decreased in the presence of higher concentrations of SWNTs, with 45±13% and 22±8% of cells remaining viable in SWNT+PEG & SWNT+COOH, respectively (Figure 1). No additional loss of cells was seen between Day 7 and Day 14.

3.2 CNT-Hydrogel Constructs (3D Culture)

The presence of carbon nanotubes altered the viability and biochemical synthesis of chondrocytes in the hydrogel composites in a dose dependent manner. No significant loss of cell viability was seen for cells cultured in the presence of 0.1 mg/ml SWNT, same concentration shown to be detrimental to cells in 2D cultures. Even at concentrations 10 fold higher, the presence of SWNT+PEG at 1.0 mg/ml resulted in limited cell death by Day 7 (Figure 2).

The GAG content of the constructs increased over the culture duration for all groups, demonstrating 2- to 4-fold increases in the control and 0.1 mg/ml CNT groups. In the presence of 1.0 mg/ml CNTs, GAG content increased significantly over all groups, demonstrating a 10- to 13-fold increases over Day 0 (Figure 3).

The compressive modulus (E_Y) of agarose ± SWNT constructs also increased with time in culture and with the addition of CNTs (Figure 4). At D0, the addition of 0.1 mg/ml SWNT+COOH increased E_Y by ~53% and that of 1.0 mg/ml SWNT+PEG by 32% (Figure 4). By D21, E_Y increased by 45% in the control group, where as the presence of 0.1 mg/ml SWNT+COOH increased E_Y by 73%. The presence of SWNT+PEG demonstrated transient effects on E_Y, with no significant increase over control (Figure 4).

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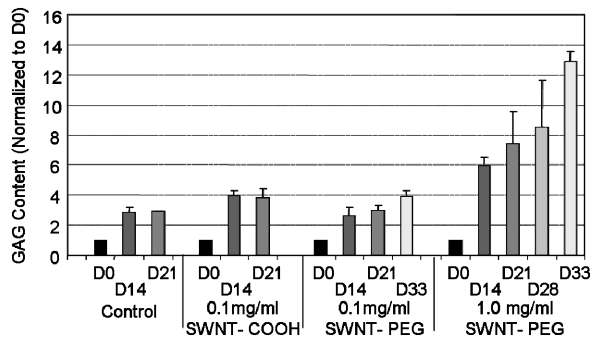

Figure 3: GAG content is composite constructs at various times in culture in the presence or absence of SWNTs (content is reported normalized to Day 0 values).

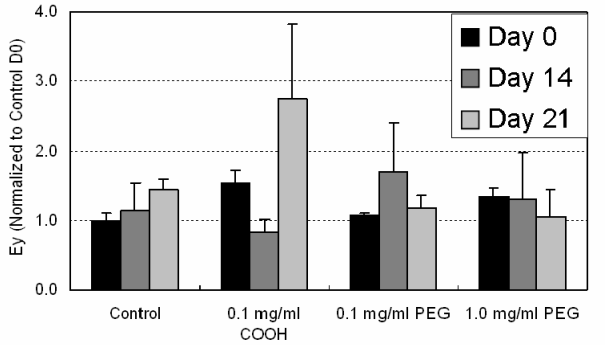

Figure 4: Young's Modulus (E_Y) of agarose constructs ± SWNTs (normalized to Day 0 control values)

Histological analysis revealed comparable GAG deposition for all groups by D21 (Figure 5). However, an increased deposition of collagen matrix was seen for chondrocytes in 1.0 mg/ml SWNT+PEG at D21. The water content of the constructs at D0 was comparable for all groups (0.89±0.04), and decreased slightly over the culture duration, reaching 0.84±0.03 by Day 21.

4 DISCUSSION

The goal of this study was to assess the long-term biocompatibility of SWNTs for chondrocyte growth. The exposure of chondrocytes to SWNTs was carried out for up to 14 days in 2D and 21 days in 3D cultures. This represents a significantly longer culture-time than previous reports examining the effects of CNTs on cell responses (such as adhesion, etc) [7]. Our results suggest that functionalized CNTs alter the viability, metabolic and mechanical environment of cells over the 2 to 3 week course of these experiments.

In 2D cultures, chondrocytes experienced a dose dependent loss in viability in the presence of SWNT+COOH or SWNT+PEG (Figure 1), where low concentrations of SWNT+PEG were as benign as sonicated medial alone (Figure 1). However, the behavior of chondrocytes in 2D can only be limitedly applicable to longer cultures, due the propensity of cells to dedifferentiate in this environment [9]. Morphological examination of cells revealed that a significant number of cells acquired fibroblast like morphology by day 14, in contrast to control samples where cells continued to maintain chondrocyte-like phenotype.

In 3D cultures, chondrocytes demonstrated viability and biochemical deposition through 21 days of exposure to SWNTs. Despite some loss of viability in the presence of SWNTs at higher concentrations, the biochemical deposition through 21 days of exposure to CNTs was found to be significantly greater than control groups (Figure 3 and 5). The presence of CNTs did not diminish the biochemical deposition of GAGs nor of collagen, compared to the control (Figure 5), suggesting that the presence of CNTs in 3D allows chondrocytes to maintain their phenotype. Moreover, 1.0 mg/ml SWNT+PEG resulted in increased deposition of collagen by Day 21. This increased collagen deposition suggests that SWNT composite structures can act as molecular sized stimulants of cellular growth and promoters of biochemical synthesis.

The presence of SWNTs also altered the biomechanical properties of these composite structures, though no obvious dose dependent behavior was observed in the equilibrium compressive modulus. The presence of SWNT+COOH increased E_Y initially (Day 0 constructs) by 53%, suggesting a reinforcement behavior prior to matrix deposition, *de novo.* Interestingly, the presence of SWNT+PEG at the same concentration did not result in a similar reinforcement behavior in compression. This finding suggests that the presence of the negatively charged carboxylic acid groups may have mediated this increase in stiffness, possibly by increasing osmotic effects. These findings are consistent with previous studies, where CNTs introduced into synthetic biopolymers were found to improve the mechanical properties of the nanocomposites [8].

Interestingly, the results of this study also suggest the dose dependent effects of CNTs on cellular responses vary between 2D and 3D cultures. Chondrocytes appear to tolerate the presence of SWNTs at 10-fold higher concentrations in 3D than concentrations shown to be lethal in 2D cultures. This may be due to the interaction of disbursed SWNTs with cellular structures, either through adhesion to the cell membrane or via endocytosis into the intracellular space. Though no specific cross links between the SWNTs and the hydrogel were targeted, the entrapment of the CNTs in the hydrogel during the curing process may have prevented cellular uptake of CNTs. These findings support the notion that optimization of the use of nanotubes or particles in cell-based therapies should be performed in the intended 3D systems directly, thus alleviating complications unique to other models in use.

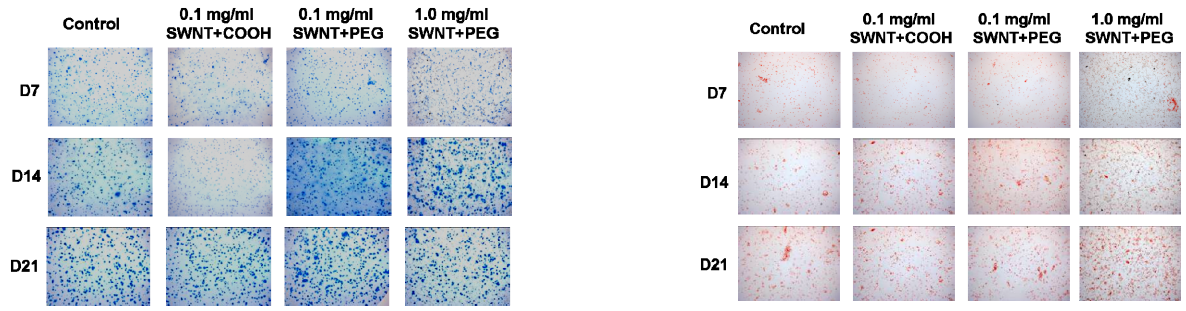

Figure 5: Histological evidence of GAG (left) and collagen (right) deposition in chondrocyte seeded agarose + SWNT composites at days 7, 14 and 21 in culture (Magnification: 10X).

In conclusion, the results of this study indicate that SWNTs offer a unique potential for cartilage tissue engineering, where functionalization with bioactive molecules may provide an ideal substrate for stimulation of cellular growth and repair. Future studies will examine the effect of growth factor conjugation to CNTs on cell growth, as well as the effect of CNTs on the tensile properties in composite structures.

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