Cardiomyocyte Function on Poly-Lactic-co-Glycolic Acid: Carbon Nanofiber Composites under Electrical Stimulation

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ABSTRACT

The present work demonstrates that a simple solution-mixing-drying based synthesis route can be adopted to develop PLGA:CNF hybrid biocomposites over a broad composition range possessing a uniform distribution of CNF without any clustering and using electrical stimulation to mimic heart conditions to promote cardiomyocyte growth. Electrical stimulation increased cardiomyocyte density on all PLGA:CNF composite ratios as well as cardiac protein biomarker Troponin I (with 50:50 [PLGA:CNF (wt:wt)] composite with the highest cell density) when compared to non-electrical stimulation data. This shows that PLGA:CNF composites can promote cardiomyocyte proliferation and differentiation in an in vitro heart model. Future work will consist of atomic force microscopy to better understand surface-cell interactions as well as surface conductivity analysis.

Keywords: carbon nanofiber, poly(lactic-co-glycolic acid), composite, carbiovascular, nanotechnology

1 INTRODUCTION

A major epidemic in the United States and worldwide is cardiovascular disease (CVD). CVD refers to a class of diseases that affect the heart and blood vessels of its host, but usually is used in relationship to atherosclerosis, or arterial disease [1, 2]. Approximately every 25 seconds, an American will have a coronary event, and approximately every minute, someone will die of one [3]. On average, every 40 seconds, someone in the United States has a stroke.[3] An estimated 74,500,000 United States' adults have hypertension with approximately 78% aware of their condition and 68% using anti-hypertensive medication, but only 44% of those treated have their hypertension controlled [3]. The total direct and indirect cost of CVD and stroke in the United States for 2010 was estimated at $503.2 billion [3]. In recent years, CVD has taken over as the number one killer of American women, surpassing breast cancer. By the time CVD has been detected, it is usually fairly advanced, having developed for decades [4, 5].

Unfortunately, no single technology offers the perfect solution. To address these problems, quick technological advancements of cell biology, genomics, and proteomics combined with discoveries in material sciences and bioengineering have created a new field of medicine, nanomedicine—utilizing materials and systems which possess at least one physical dimension between 1-100 nm to construct structures, devices, and systems that have novel properties [6].

The advent of advanced novel nano-biomaterials with improved properties capable of being used in several biomedical applications simultaneously, has transformed the field of biomedical research. Nanomaterials are among the most intensively studied materials for a wide range of applications ranging from fuel cells [7] to nanopatches for the myocardium [8, 9]. These nanostructured composite materials are combinations of at least two constituent materials, a matrix (host) and a reinforcement component (i.e. nanofiller) [10]. It is known that the properties of materials change considerably when the size of constituents is significantly small, within the 1–100 nm size range [11]. Since these materials have improved physical, chemical, and mechanical properties, they are significantly versatile for a wide range of applications.

One such example is the use of a poly(lactic-co-glycolic acid) (PLGA) composite with embedded carbon nanofibers (CNF) to create a "patch" for an infarcted area of the heart. Recent research has shown that when one adds CNFs to PLGA, cardiomyocytes (specific heart tissue cells responsible for contraction) will adhere and proliferation better than on their non-nanoreinforced counterparts, but the mechanism is still largely unknown [9].

To further understand cardiomyocyte function on such novel materials, the purpose of this present in vitro study was to alter the CNF density of the PLGA:CNF composite to determine if CNF density has an effect on a nano-inspired cardiovascular patch, both in a static and electrically stimulated state, while analyzing cardiomyocyte function, via the Troponin complex (the regulatory proteins that are integral to muscle contraction in myocardium [12, 13]), and if so, what density would promote cardiomyocyte proliferation the greatest.

2 METHODS AND MATERIALS

2.1 PLGA:CNF Composite Synthesis and Analysis

The fabrication procedure for the PLGA:CNF composites have been detailed in previous studies. In short, a PLGA density of 0.025 g/ml (50:50 PLA:PGA wt.%; Polysciences Cat #23986) were created by dilution in a 50

ml flask with 30 ml of tetrahydrofuran (THF; Mallinckrodt Chemicals Lot #C45763) and were sonicated in a water bath (VWR B3500A-DTH) below 30^0C for 30 minutes. 500 mg of CNFs (99.9% by weight %, Catalytic Materials, MA) with a diameter of 100 nm of different lengths, from 100 to 200 microns, were sonicated (Misonix Sonicator 3000) in a 50 ml beaker with 20 ml of chloroform (Fisher Science Lot #102591) at 20W for 30 min. After obtaining the separately sonicated PLGA and CNF solutions, various PLGA:CNF weight percent ratios were developed by altering the CNF material weight density (100:0, 75:25, 50:50, 25:75, and 0:100) by adding the appropriate amount of CNF to PLGA in 20 ml disposable scintillation vials. The CNF weight ratios were measured using a laboratory balance (Mettler Toledo AL54). When the appropriate ratios were added, each composite material was sonicated (Misonix Sonciator 3000) at 10W for 20 minutes each.

For experimental ease, a 22 mm diameter microscope cover glass (Fisher Science circles No. 1 - 0.13 to 0.17mm thick; Size: 22 mm, Cat #12-545-101) was coated with the aforementioned composite. Before the PLGA:CNF composite was positioned onto the glass substrate, the glass substrate was cleaned via soaking in a 70:30 (vol. %) ethanol-deionized solution shaking (VWR, Advanced Digital Shaker) for 10 minutes. Next, the substrate was added to a 100% deionized water solution and shaken for 10 minutes.

Using a disposable pipette (Fisherbrand #13-711-9AM), 1 ml of the appropriate PLGA:CNF composite solution was placed onto the glass substrate and placed into an oven at 42^0C for 15 minutes. Each composite film was then vacuum dried (Shel Lab) at 20 inches of Hg gauge vacuum pressure for 48 hours to allow the tetrahydrofuran (THF) and chloroform to evaporate. All the samples and controls were sterilized using ultraviolet light for 24 hours prior to cell seeding.

2.2 Electrical Stimulation and Cytocompatibility

For in vitro analysis, human cardiomyocytes (Celprogen, Cat #36044-15) were seeded at a cell density of 10 x 10^4 cells/cm^2 for the cell adhesion assay and 10 x 10^4 cell/cm^2 for the cell proliferation assay on PLGA:CNF composites in complete growth media supplemented with 10% fetal bovine serum and 1% antibiotics (Celprogen, Cat #M36044-15S). Cells were seeded onto 12-well human cardiomyocyte stem cell culture extra-cellular matrix plates (Celprogen, Cat #E36044-15-12Well) on top of the various PLGA:CNF samples, and 22 mm diameter microscope cover slips (Fisher Science circles No. 1 - 0.13 to 0.17mm thick; Size: 22 mm, Cat #12-545-101) were used as controls. The samples were incubated for 24, 72, and 120 hours for the proliferation assay under standard incubation conditions (at 5% CO_2 95% humidified air and 37^0C, changing the media every other day).

For in vitro electrical stimulation analysis, human cardiomyocytes (Celprogen, Cat #36044-15, USA) were seeded onto PLGA:CNF composites (prepared as stated above) in complete growth media supplemented with fetal bovine serum and 1% antibiotics (Celprogen, Cat #M36044-15S, USA) at a density of 10 x 10^4 cells/cm^2 and were continuously stimulated (rectangular, 2 nm, 5 V/cm, 1 Hz) with a C-Pace EP Culture Pacer (IonOptix LLC, USA) for 24, 72, and 120 hours to mimic normal heart performance [14, 15]. Troponin I (Calbiotech, Cat #TI015C, USA) enzyme-linked immunosorbent assays (ELISA) were completed in tandem to analyze cardiomyocyte viability and protein synthesis on the composites.

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Progema, CellTiter 96® Non-Radioactive Cell Proliferation Cat. #G-4100) assays were completed to analyze cytocompatibility and cell viability on the composite.

Cardiomyocyte proliferation and Troponin I assays were performed at least in triplicate with three repeats each and results were compared to a control glass surface. The optical density (OD) data for cardiomyocyte MTT assays were plotted as mean ± standard error of the mean while OD to cell count conversions were conducted by using a stand curve analysis between OD and cell numbers. Statistical analyses were performed. When data were compared, ANOVA software and a student T-test were used. A p-value of < 0.05 was considered to be significant.

3 RESULTS AND DISCUSSION

3.1 PLGA:CNF Composite Synthesis and Analysis

A SEM image of the as-synthesized PLGA:CNF composite surface are shown in Figure 1c. CNFs were uniformly dispersed within the PLGA matrix and, as expected; more CNFs were observed for the higher CNF ratio samples. When looking at the different PLGA densities and similar CNF ratios, uniform CNFs were again uniformly dispersed within the PLGA matrix, suggesting that materials were properly generated (Figure 1).

XRD spectra obtained from the as-synthesized PLGA:CNF composites are shown in Fig. 1a. As expected an extremely broad and flat peak was recorded for the PLGA matrix, confirming its amorphous nature. However, with addition of CNF in PLGA, the characteristic X-ray peak at $2\Theta = 26.5°$ evolved into a rather strong and sharp peak in PLGA:CNF composites for the 25:75 [PLGA:CNF (wt:wt)] ratio. The curves were fitted to the characteristic peak by Gaussian distribution. Clearly, the intensity increased as full width at half maximum (FWHM) decreased as CNFs were added to PLGA (Fig. 1b).

3.2 Material Cytocompatibility

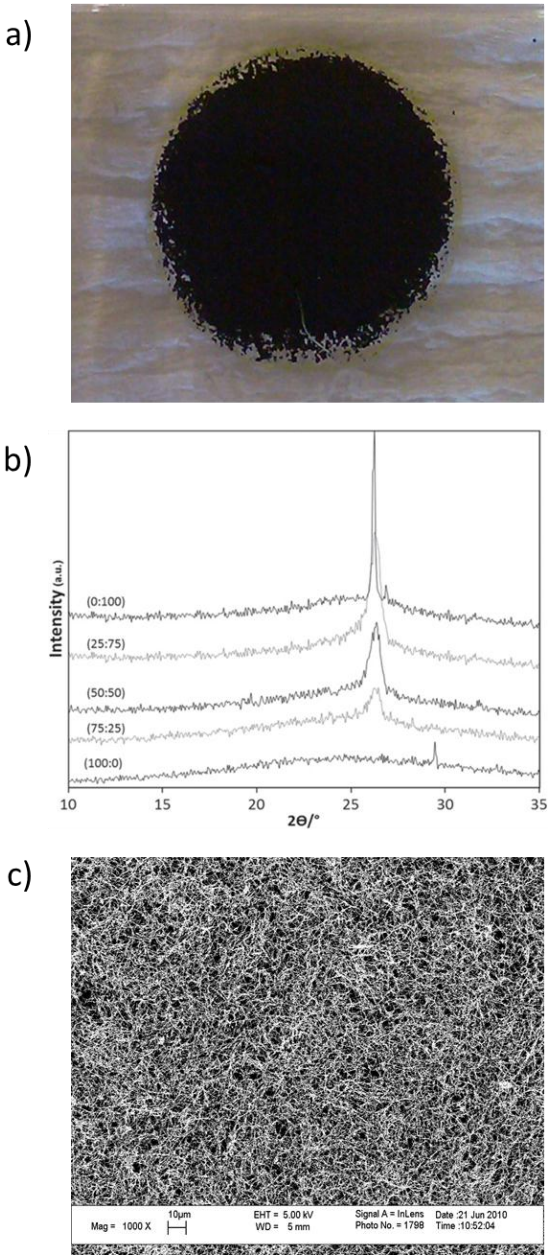

Figure 1: Material analysis of a 50:50 [PLGA:CNF (wt:wt)] composite: a) depiciting a visual representation of composite on a 22 mm diameter microscope cover glass after all synthesis steps were completed; b) X-ray diffraction results of all composites with varying CNF densities; c) scanning electron micrograph at 1K magnification showing the distribution of CNFs in a PLGA matrix Scale bar = 10 µm.

For cardiomyocyte proliferation experiments, results indicated that PLGA:CNF composites promoted human cardiomyocyte proliferation more when electrically stimulated compared to pure PLGA. General trends were seen between each ratio and supported published results [9]. For 100nm diameter CNFs, it was determined that the 50:50 ratio [PLGA:CNF; (wt:wt)] had the highest cardiomyocyte density at all three time points, whereas the lowest density was observed on the 100:0 ratio sample [PLGA:CNF; (wt:wt)] (Fig. 2). Statistical analysis using ANOVA showed that the cardiomyocyte results were significant at the 5% significance level for both adhesion and proliferation assays for all densities and composite ratios. Due to the slower proliferation of the cardiomyocytes (Celprogen, Cat #36044-15) compared to other cells (around 72 hours for cell doubling times), one would not see a typical doubling time every 48 hours.

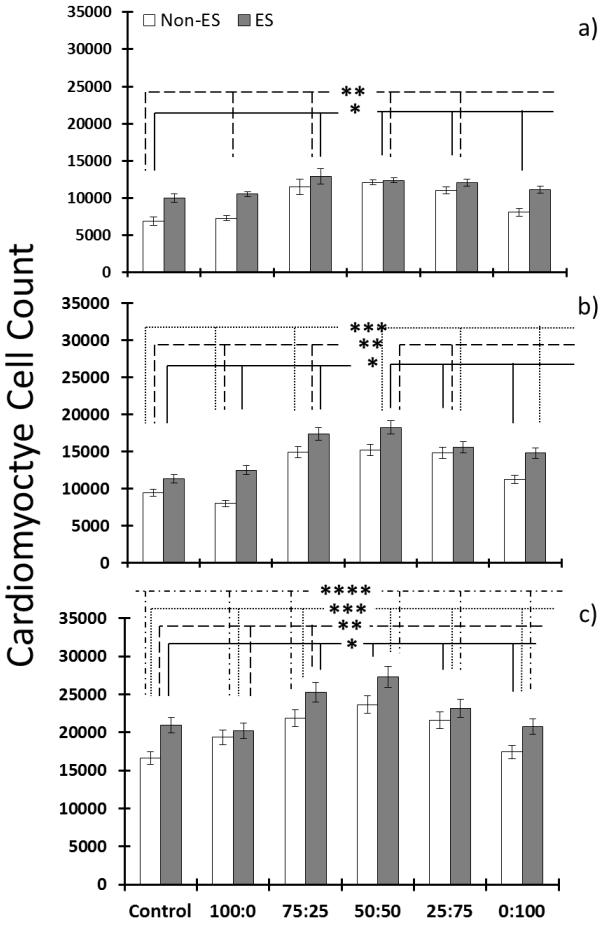

Figure 2: Cardiomyocyte cell proliferation between non-electrical stimulation (Non-ES) compared to electrical stimulation (ES) after a) 24, b) 72 and c) 120 hrs on 100 nm diameter PLGA:CNF composite materials of interest to this study. Seeding density = 10 x 104 cells/cm2. Data are mean counts ± s.d. to n=3. Control was a glass substrate. * $p < 0.05$ compared to the control, ** $p < 0.05$ compared to similar PLGA:CNF composite ratios and different densities. *** $p < 0.05$ compared to similar PLGA:CNF composite ratios between 24 and 72 hrs. **** $p < 0.05$ compared to similar PLGA:CNF composite ratios between 72 and 120 hrs.

Looking at cardiomyocyte function, Troponin-I assays indicated that cardiomyocytes had a functional Troponin Complex throughout the cytocompatibility assays

It is worth speculating why greater cardiomyocyte attachment and growth were observed on such composites with increasing CNF density in PLGA. In the past, researchers have found that the adsorption and bioactivity of vitronectin increases on nanophase ceramics and that this enhanced osteoblast functions (including adhesion, proliferation, and differentiation); these studies reported that the roughness was closer to the nanometer roughness of bone in nano-ceramics, and that led to more bone growth on nanophase ceramics [16].

Clearly, CNFs possess nanoscale geometries which imitate the extracellular matrix of various tissues (such as the heart), potentially leading to improved cytocompatibility properties of these materials [17]. Although requiring further study, nanotechnology (or the use of CNFs) can play a similar important role towards promoting cardiomyocyte density by increasing platelet-derived growth factor-BB (PDGF-BB) adsorption, which in turn, will induce cardiomyocyte adhesion and proliferation [18].

4 CONCLUSIONS

The present work demonstrates that a simple solution-mixing-drying based synthesis route can be adopted to develop PLGA:CNF hybrid biocomposites over a broad composition range possessing a uniform distribution of CNF without any clustering and using electrical stimulation to mimic heart conditions to promote cardiomyocyte growth. Electrical stimulation increased cardiomyocyte density on all PLGA:CNF composite ratios as well as cardiac protein biomarker Troponin I (with 50:50 [PLGA:CNF (wt:wt)] composite with the highest cell density) when compared to non-electrical stimulation data. This shows that PLGA:CNF composites can promote cardiomyocyte proliferation and differentiation in an in vitro heart model. Future work will consist of atomic force microscopy to better understand surface-cell interactions as well as surface conductivity.

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