The Effect of Carbon Nanofiber Density in Poly(lactic-co-glycolic acid) Composites for Myocardial Tissue Engineering Applications

David A. Stout[1,2,a] and Thomas J. Webster[1,3,b]

[1]School of Engineering, Brown University, 182 Hope Street Box D, Providence, RI 02912, USA
[2]Division of Biology and Medicine, Brown University, Box G-A, Providence, RI 02912, USA
[3]Department of Orthopaedics, Brown University, 593 Eddy Street, Providence RI 02903, USA
[a]david_stout@brown.edu, [b]th.webster@neu.edu

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ABSTRACT: Recent research (in vitro) has shown that one can increase cardiomyocyte adhesion and proliferation using poly(lactic-co-glycolic acid) (PLGA) (50:50 wt.%) supplemented with carbon nanofibers (CNFs). To better understand the promoted growth of cardiomyocytes, different PLGA: CNF composites were created where different CNF densities were tested (ranging from 0-100% CNFs) with the notation 100:0, 75:25, 50:50, 25:75, and 0:100 [PLGA:CNF (wt:wt)] for cytocompatibility properties under electrical stimulation with human cardiomyocytes. X-Ray diffraction spectra obtained from the as-synthesized PLGA:CNF composites showed an extremely broad and flat peak recorded for the PLGA matrix, confirming its amorphous nature and characteristic X-ray peak at $2\Theta = 26.5°$ which evolved into a rather strong and sharp peak with the addition of CNF. Results indicated that cardiomyocyte cell density increased with continuous electrical stimulation (rectangular, 2 nm, 5 V/cm, 1 Hz) after 24, 72, and 120 hours as well as a slight increase in Troponin I excretion compared to non-electrically stimulated normal cardiomyocyte cell functions. Furthermore, the results specified that CNF density did have an effect on PLGA:CNF composite cytocompatibility properties with the best results coming from the 50:50 [PLGA:CNF (wt:wt)] composite. This study, thus, provides an alternative conductive scaffold using nanotechnology which should be further explored for numerous cardiovascular applications.

KEYWORDS: nanotechnology, cardiovascular, cardiomyocyte, carbon nanofibers.

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 State's 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].

Although changes in environmental exposure, reduction in tobacco use, adjustments in diet, and increased physical activity can all improve patient health, the progression of CVD in the world is growing. New models to detect and treat CVD in asymptomatic patients are needed in order to prevent the first symptoms from being the last as a result of death. A need for improved and safer approaches to coronary and intracranial revascularization is still required, even with advances in the last 10 years.

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, 7].

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 [8, 9] to nanopatches for the myocardium [10, 11]. These nanostructured composites are combinations of at least two constituent materials, a matrix (host) and a reinforcement component (i.e. nanofiller) [12]. 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 [13-15]. Since these materials have improved physical, chem-

ical, 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 (Fig. 1). Recent research has shown that when one adds CNFs to PLGA, cardiomyocytes (specific heart tissue cells responsible for contraction) will adhere and proliferate better than on their non-nanoreinforced counterparts, but the mechanism is still largely unknown [11].

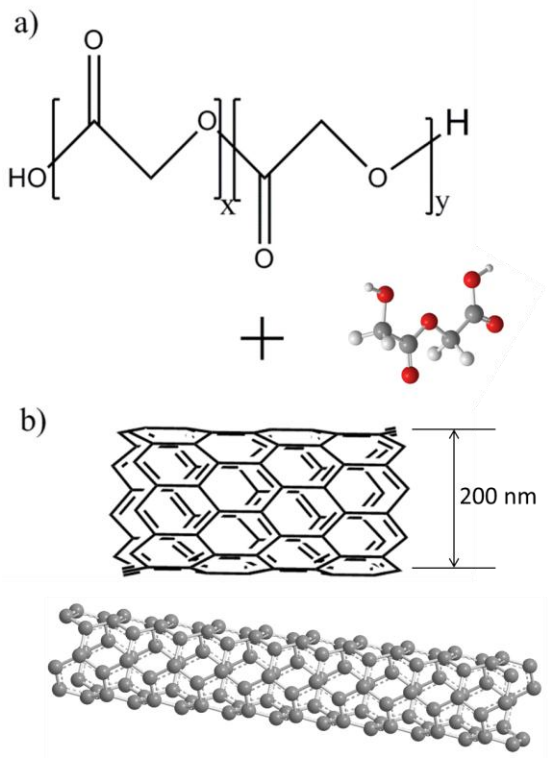

Figure 1. Chemical structure of a) poly(lactic-co-glycolic acid) (PLGA) and b) a carbon nanofiber (CNF). Shapes were created in in Chem3D Pro 12.0 (PerkinElmer Informatics).

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 (Fig. 2), while analyzing cardiomyocyte function via the Troponin complex (the regulatory proteins that are integral to muscle contraction in the myocardium [16-18]) (Fig. 3). Lastly, it was important to determine what density would promote cardiomyocyte proliferation the greatest.

2. Methods

The fabrication procedure for the PLGA:CNF composites have been detailed in previous studies [11]. In short, a PLGA density of 0.025 g/ml (50:50 PLA:PGA wt.%; Polysciences Cat #23986) was 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^{0}C for 30 minutes. 500 mg of CNFs (99.9% by weight %, Catalytic Materials, MA) with a diameter of 100 nm and 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 20 W 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.

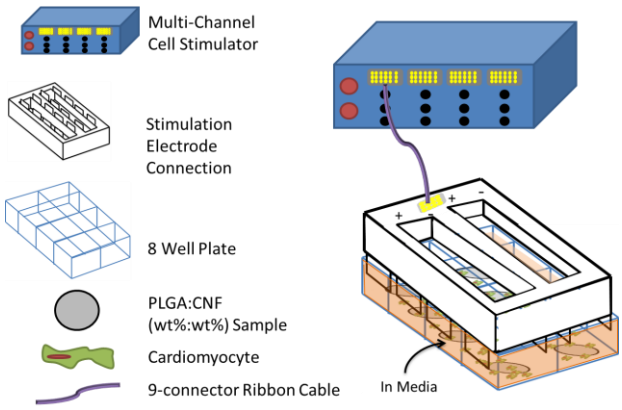

Figure 2. Schematic of the electrical stimulation (ES) apparatus (C-Pace EP Culture Pacer (IonOptix LLC, USA)) and experimental design. Human cardiomyocytes were seeded onto PLGA:CNF composites at a density of 10 x 10^4 cells/cm^2 and were continuously stimulated (rectangular, 2 nm, 5 V/cm, 1 Hz) for 24, 72, and 120 hours.

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^{0}C 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.

A Hitachi 2700 scanning electron microscope was used to characterize the surface of the PLGA:CNF samples at 5 KV with an InLens system and software. X-Ray diffraction (Bunker AXS: D8 Focus) was used with settings at 0.5 ° per 2 minutes between 2Θ = 10-35 ° to characterize the crystallinity of the composites.

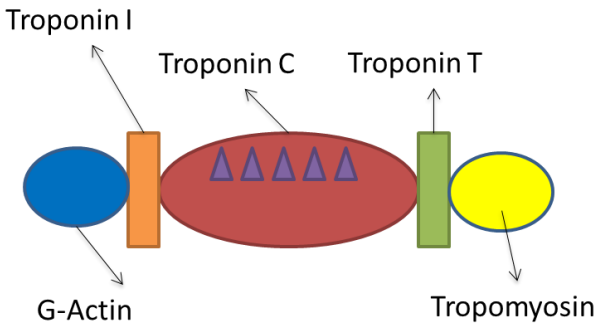

Figure 3. A schematic of the Troponin complex which binds to actin in thin myofilaments to hold the actin-tropomyosin complex in place. Troponin consists of three subunits: Troponin C (TnC) which is the Ca^{2+}-binding subunit; Troponin I (TnI), which is the the inhibitory subunit; and Troponin T (TnT) which is the tropomyosin-binding subunit [19]. Tropomyosin is a α-helical coiled-coil protein throughout its entire length, and interacts with adjacent tropomyosin molecules in a head-to-tail manner, forming continuous strands that lie along the thin filament [16]. When calcium binds to the Troponin C, it causes conformational changes which lead to dislocation of Troponin I and finally tropomyosin leaves the binding site for myosin on actin leading to contraction of muscle.

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 [20, 21]. 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 the mean ± standard error of the mean while OD to cell count conversions were conducted by using a standard curve analysis between OD and cell numbers. 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 discussions

3.1 PLGA:CNF characteristics

An SEM image of the as-synthesized PLGA:CNF composite surface is shown in Figure 4c. CNFs were dispersed within the PLGA matrix and, as expected, more CNFs were observed for the higher CNF 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 (Fig. 4).

XRD spectra obtained from the as-synthesized PLGA:CNF composites are shown in Fig. 4a. As expected an extremely broad and flat peak was recorded for the PLGA matrix, confirming its amorphous nature. However, with the addition of CNF to PLGA, the characteristic X-ray peak at 2Θ = 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. 4b).

3.2 PLGA:CNF cytocompatiblity

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 [11].

For the 100 nm 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. 5). 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.

Looking at cardiomyocyte function, Troponin-I assays indicated that cardiomyocytes had a functional Troponin Complex throughout the cytocompatibility assays. All CNF experiments for all time points showed a slight increase of Troponin-I indicating better productivity [22]. As discovered, the Troponin-I assays presented the same trends seen in the proliferation cytocompatibility assays (Fig. 6)

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 function (including adhesion, proliferation, and differentiation) was due to roughness values closer to the nanometer roughness of bone itself [23].

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 [24]. 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 [25].

While the mechanism of enhanced cardiomyocyte density is still not thoroughly known at this time, it could have to do with the topography of PLGA:CNF composites which dictates surface energy to control protein adsorption events. Importantly, it is also known that a material can be too rough and can hinder cellular activity [26]. Yang et al. showed that diamond films with nanometer and micron scale topographies, fabricated through microwave plasma enhanced chemical-vapor-deposition and hydrogen plasma treatment, influenced osteoblast adhesion and proliferation, with the nano-sized diamond topographies better than micron topographies [26].

Even though the mechanism is not fully understood, it is shown here that CNF density does play a role in mediating cardiomyocyte growth and functionality. This may be due to the creation of a material which resembles normal heart tissue material characteristics better (such as topography, conductivity, mechanical properties, etc.). Altering CNF density in the composites was able to provide a more conducive environment for cardiomyo-cyte functions.

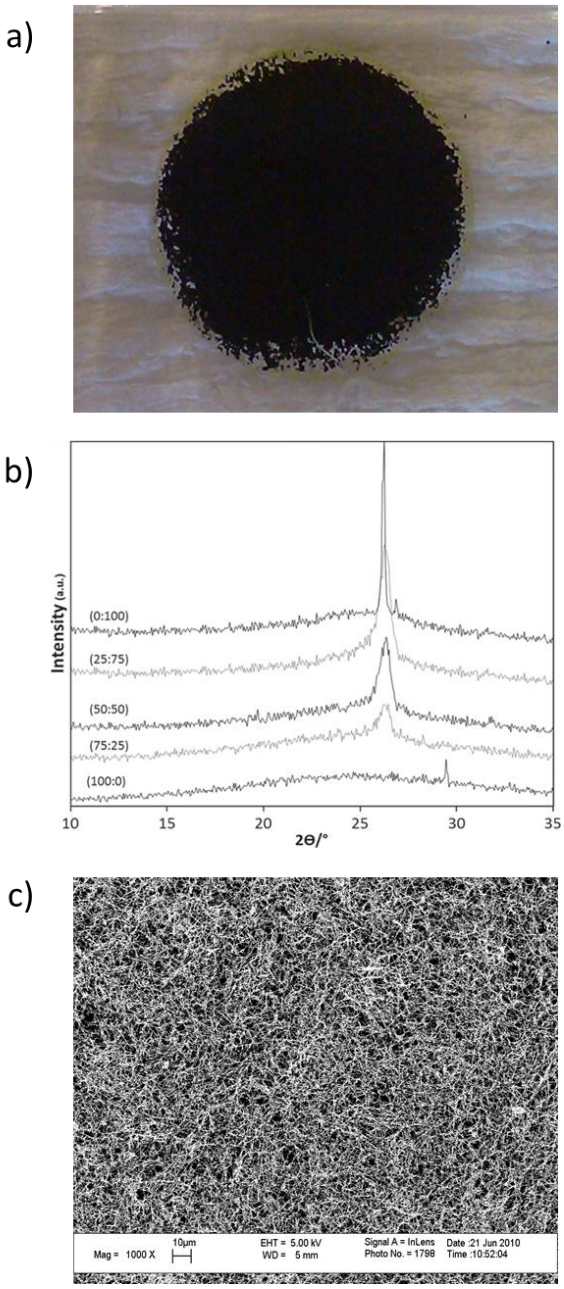

Figure 4. Material analysis of a 50:50 [PLGA:CNF (wt:wt)] composite: a) depicting a visual representation of composite on a 22 mm diameter microscope cover glass (Fisher Science circles No. 1 - 0.13 to 0.17 mm thick; Size: 22 mm, Cat #12-545-101) after all synthesis steps were completed; b) X-ray diffraction 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.

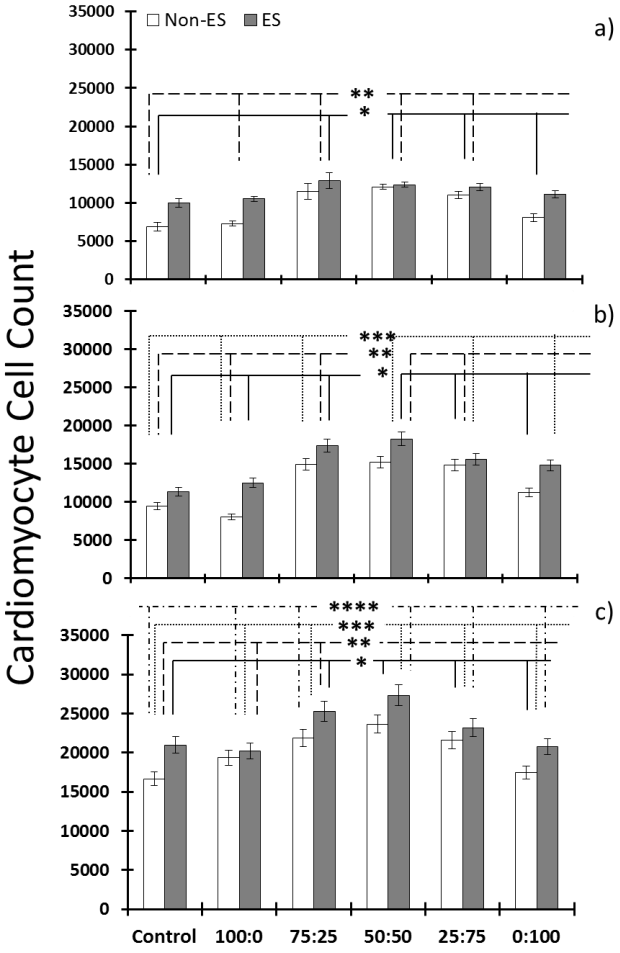

Figure 5. Cardiomyocyte cell proliferation between non-electrical stimulation (Non-ES) and electrical stimulation (ES) after a) 24, b) 72 and c) 120 hrs on 100 nm diameter PLGA:CNF composite materials of interest to this study. Seeding density = 10 x 10^4 cells/cm^2. Data are mean counts ± s.d. to n=3. 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.

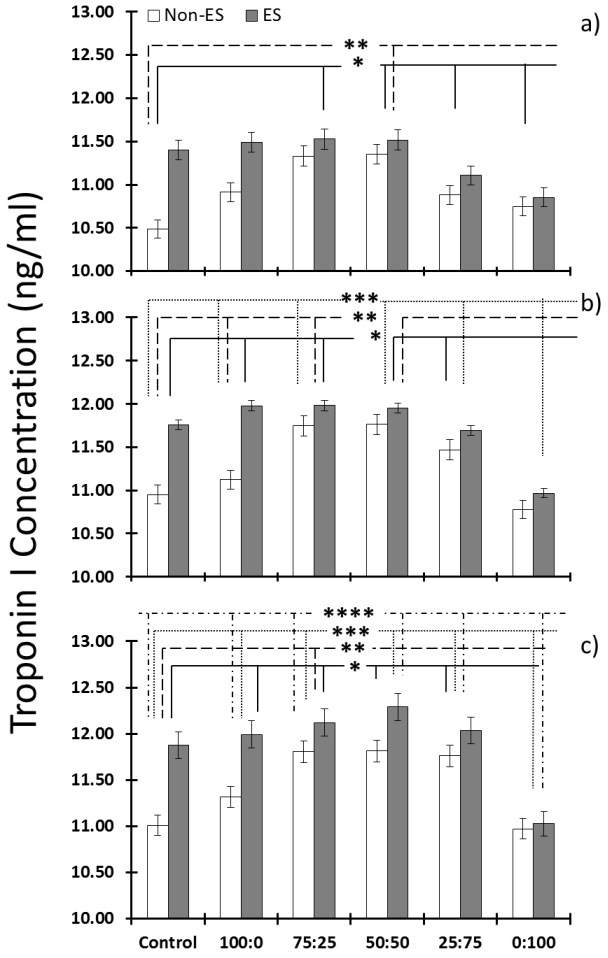

Figure 6. Comparison of Troponin I concentration from cardiomyocyte cell proliferation between non-electrical stimulation (Non-ES) and electrical stimulation (ES) after a) 24, b) 72 and c) 120 hrs on 100 nm diameter PLGA:CNF composite materials of interest to this study. Seeding density = 10 x 10^4 cells/cm^2. Data are mean counts ± s.d. to n = 3. Control was a glass substrate. * $p < 0.05$ compared to the control of all Non-ES samples, ** $p < 0.05$ compared to the control of all ES samples. *** $p < 0.05$ 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.

4. Conclusions

In the present work it was demonstrated that a simple solution-mixing-drying based synthesis route can be adopted to develop PLGA:CNF hybrid biocomposites over a broad CNF density range possessing a uniform distribution of CNF without any clustering and using electrical stimulation to mimic heart conditions to promote cardiomyocyte growth. With the use of an electrical stimulation system we were able to demonstrate increased cardiomyocyte density on all PLGA:CNF composite ratios as well as cardiac protein biomarker Troponin I (with the 50:50 PLGA:CNF (wt:wt)) composite with the highest cell density and Troponin I levels) when compared to non-electrical stimulation data as well normal glass substrates. These results indicated that CNF density does have an effect on PLGA:CNF composite cytocompatibility and should be further studied for myocardial tissue engineering applications, where specifically 100 nm CNF 50:50 PLGA:CNF (wt:wt) composites would be the best of those studied.

Acknowledgments

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