Electrically Conductive Chitosan/Carbon Scaffolds for Cardiac Tissue Engineering

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ABSTRACT: In this work, carbon nanofibers were used as doping material to develop a highly conductive chitosan-based composite. Scaffolds based on chitosan only and chitosan/carbon composites were prepared by precipitation. Carbon nanofibers were homogeneously dispersed throughout the chitosan matrix, and the composite scaffold was highly porous with fully interconnected pores. Chitosan/carbon scaffolds had an elastic modulus of 28.1 ± 3.3 kPa, similar to that measured for rat myocardium, and conductivity of 0.25 ± 0.09 S/m. The scaffolds were seeded with neonatal rat heart cells and cultured for up to 14 days, without electrical stimulation. After 14 days of culture, the scaffold pores throughout the construct volume were filled with cells. The metabolic activity of cells in chitosan/carbon constructs was significantly higher as compared to cells in chitosan scaffolds. The incorporation of carbon nanofibers also led to increased expression of cardiac-specific genes involved in muscle contraction and electrical coupling. This study demonstrates that the incorporation of carbon nanofibers into porous chitosan scaffolds improved the properties of cardiac tissue constructs, presumably through enhanced transmission of electrical signals between the cells.

INTRODUCTION

Cardiac muscle is an electroactive tissue capable of transferring electrical signals and allowing the heart to beat. When damaged, adult heart muscle has poor capability to repair itself due to a minimal regeneration potential of cardiomyocytes. In the past decade, great interest has arisen from the possibility to regenerate lost tissue by implanting therapeutic cells, biomaterials, and cardiac patches. Selection of a scaffold with appropriate mechanical and electrical properties is critical for inducing functional cardiac tissue, in vitro or in vivo. Several natural-based scaffolds have been proposed for cardiac applications and studied with cells with the application of mechanical and electrical stimulation. The development of conductive materials for cardiac regeneration was also reported. Chitosan is a suitable functional biomaterial because it is biocompatible, biodegradable, minimally immunogenic, nontoxic, and hydrophilic. In addition, chitosan is a hemostatic agent, with antithrombogenic properties. Chitosan-based composites can combine several properties of interest to cardiac tissue engineering, including the natural-based origin and biodegradability (like collagen scaffolds) with adequate mechanical properties and electrical conduction. Since chitosan is nonconductive, its electrical properties could be improved by adding conductive material. We selected carbon nanofibers over carbon nanotubes for two reasons. Carbon fibers are larger in size and therefore easier to incorporate into chitosan. Also, carbon nanofibers have more sites on the outer wall than carbon nanotubes, which can facilitate the electron transfer of electroactive analytes, such as proteins or enzymes.

Carbon nanofibers have ability to reinforce polymer scaffolds and produce excellent mechanical properties. Also, carbon nanostructures have been considered as reinforcing filler for biological matrices to improve multiple functions, including electrical conductivity. Chitosan has been reinforced with carbon nanotubes to form various types of composites for biological applications but this material was never investigated for cardiac tissue engineering.

We hypothesized that electrically conductive scaffolds would improve cardiomyocyte function by increasing expression of cardiac genes, even without electrical stimulation. Our strategy was to combine the biocompatibility and biodegradability of

Received: November 13, 2013
Revised: January 10, 2014
Published: January 13, 2014
chitosan with the electrical properties of carbon nanofibers. By seeding neonatal cardiomyocytes into chitosan/carbon scaffolds in the absence of electrical stimulation we investigated the following questions: (i) Are chitosan/carbon scaffolds adequate materials for cardiac applications, in terms of their mechanical and electrical properties? (ii) Is the integrity of chitosan/carbon scaffolds maintained over time in culture? (iii) What are the effects of carbon nanofibers on metabolic activity of cultured cardiomyocytes and the expression of cardiac genes? In this initial study, we found that chitosan/carbon scaffolds support the cultivation of cardiomyocytes over a period of two weeks, with increased metabolic activity and pronounced expression of specific cardiac genes associated with contraction and the conduction of electrical signals.

**EXPERIMENTAL SECTION**

**Scaffold Preparation.** The precipitation method\(^1\),\(^2\),\(^3\) was used to prepare scaffolds containing either 2% chitosan (w/v) or 2% chitosan (w/v) and 1% carbon nanofibers (w/v). All reagents were purchased from Sigma (St. Louis, MO) unless otherwise specified. Briefly, chitosan was dissolved in 1% (v/v) acetic acid to obtain a 2% (w/v) solution. Another formulation was prepared from a solution containing 2% chitosan (w/v) and 1% carbon nanofibers (w/v; Cat. 719781, graphitized (iron-free), composed of conical platelets, D × L = 100 nm × 20–200 μm; w/v). The chitosan solution and chitosan/carbon dispersion were cast into Petri dishes and frozen immediately at −20 °C, a temperature that was maintained for 12 h.\(^4\) They were then immersed in a precipitation solution that was formed by mixing 25% (v/v) NaOH 1 M and 75% (v/v) Na\(_2\)SO\(_4\) 0.5 M. The resulting scaffolds were washed with distilled water and lyophilized.

![Figure 1. Scaffold preparation. Chitosan scaffolds were prepared using a 2% (w/v) solution of chitosan in 1% (v/v) acetic acid. An additional 1% carbon (w/v) was dispersed in the solution to make chitosan/carbon scaffolds. The solutions were casted into Petri dishes, frozen (−20 °C) overnight and immersed in a precipitation solution containing 25% (v/v) NaOH 1 M and 75% (v/v) Na\(_2\)SO\(_4\) 0.5 M. The resulting scaffolds were washed with distilled water and lyophilized.](image)

Degradation studies were performed by incubating the chitosan and chitosan/carbon scaffolds (n = 3 per group and time point) in phosphate buffered saline (PBS; pH 7.4) under static and dynamic conditions (agitation at 60 rpm) for up to 60 days. At each time point, the samples were removed, weighted for determination of water uptake, and dried for calculation of the weight loss.

**Electrical Conductivity.** The electrical conductivity of the dry and hydrated scaffolds was measured at room temperature using a Keithley 487 Picoammeter–Voltage source. The voltage was varied from −0.01 to 0.01 V in steps of 0.001 V at 1 s intervals, and the current was measured within a Faraday cage, to avoid electromagnetic field interference. The volume resistivity (ρ) was then calculated from the characteristic I–V curves by taking into account the geometrical characteristics of the sample:

\[
\rho = \frac{V}{A/L}
\]

where V is the applied voltage, I is the measured current, A is the area of the electrodes, and L is the distance between electrodes, corresponding to the thickness of the sample. In the present case the area was 19.6 \(×\) 10\(^{-4}\) m\(^2\) and the sample thickness was 3–4 mm.

Electrical conductivity (σ) was then calculated as the inverse of the resistivity (ρ):

\[
\sigma = \frac{1}{\rho}
\]

**Culture of Neonatal Rat Cardiomyocytes.** Neonatal rat cardiomyocytes were isolated from 2 day old Sprague–Dawley rats...
(Harlan, South Easton, MA), as previously described\(^6\) according to an institutionally approved IACUC protocol. Briefly, heart ventricles were quartered, incubated at 4°C in a 0.06% (w/v) trypsin in Hank’s Balanced Salt Solution (HBSS, Gibco, Carlsbad, CA), and subjected to a series of digestions (3–4 min, 37°C, 150 rpm) in a 0.1% (w/v) solution of collagenase type II (Worthington Biochemical Corp., Lakewood, NJ) in HBSS. Cell suspensions from the first 4–5 digestions were collected and preplated in polystyrene culture flasks for 75 min to remove the readily adhering cardiac fibroblasts. The nonadherent cells, that are enriched for cardiac myocytes, were counted using a hemocytometer and suspended in cardiac medium (CM) consisting of high glucose Dulbecco’s Modified Eagle’s Medium (DMEM, Gibco) supplemented with 10% heat inactivated fetal bovine serum, as previously described.

Scaffolds (6 mm diameter × 1.5 mm thick discs) were hydrated in culture medium in six-well plates and seeded with neonatal rat cardiomyocytes (5 × 10\(^6\) cells in 25 μL Matrigel (Becton Dickinson, Franklin Lakes, NJ), as described previously.\(^7\) After 45 min, 6 mL of culture medium was added to each well. High glucose Dulbecco’s modified Eagle’s medium (DMEM) was used, supplemented with 10% heat-inactivated fetal bovine serum (FBS; Invitrogen, Carlsbad, CA, U.S.A.), 1% HEPES, and 1% penicillin/streptomycin (P/S). The medium was changed every day. After 7 and 14 days, cell/constructs were removed from plates and analyzed.

**Metabolic Activity.** Cellular metabolism was evaluated using 3-((4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2(4-sulfophenyl)-2H-tetrazolium (MTS) assay (Promega; n = 4). Optical density (O.D.) was calculated as the difference between the measured value of the cell-seeded groups and the cell-free controls. Optical density values from MTS assay were assessed after 7 and 14 days of culture with neonatal cardiomyocytes on chitosan and chitosan/carbon scaffolds.

**Scanning Electron Microscopy (SEM).** Scanning electron microscopy (SEM) was used to evaluate the scaffold morphology and infiltration of the cells. At timed intervals, constructs were removed from the plates, washed in PBS, and fixed with 2.5% glutaraldehyde for 30 min. The samples were then washed with PBS, dehydrated in a graded series of ethanol, dried by hexamethyldisilazane, mounted on aluminum stages, sputter coated with gold (Fisons Instruments, Sputter Coater SC502, U.K.), and examined with an a Leica Cambridge S360 Scanning electron microscope.

**Histological Evaluation.** The remaining scaffold/cell constructs were rinsed with PBS, fixed in 4% paraformaldehyde solution embedded in paraffin, sectioned perpendicular to the scaffold diameter in 5 μm thick cross sections and stained with hematoxylin and eosin (H&E) for histological evaluation. Sections were visualized using Olympus IX81 light microscope (Center Valley, PA).

**Gene Expression.** The expression profiles of specific-cardiac markers: Atrial natriuretic factor (Anf or Nppa); α-myosin heavy chain (Myh6); β-myosin heavy chain (Myh7); Troponin T Type 2 (Tnnt2); Troponin C Type 1 (Tnc1); Gata binding protein 4 (Gata4); Gap junction protein, α-1 or connexin-43 (Gja1 or Cx43); and calcium-transporting ATPase (Atp2a2 or Serca-2) were analyzed by real-time quantitative PCR.

Cell constructs were harvested after 7 and 14 days of culture to evaluate the RNA levels of cardiac genes. Constructs were disintegrated in 1 mL of Trizol using steel beads and a bead beater rotator MiniBeadBeater-8 (Biospec Products, Bartlesville, OK), and RNA was extracted using Trizol reagent according to the manufacturer’s instructions. RNA quantity and purity were assessed using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, U.S.A.). Samples with a 260:280 ratio of 1.6 and 2.0. cDNA were prepared using Ready-To-Go You-Prime First-Strand Beads kit (GE Healthcare Life Sciences) according to the manufacturer’s instructions. Primers and glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) were purchased from Applied Biosystems (Foster City, CA, U.S.A.).

Real-time quantitative PCR (real time-qPCR) analysis was performed on the ABI Prism 7700 Sequence Detection Instrument (Applied Biosystems, Foster City, CA, U.S.A.). The Livak method (\(\Delta\Delta CT\))\(^8\) was used to quantitate relative and absolute expressions of each gene between chitosan/carbon scaffolds and chitosan scaffolds. Expression of cardiac specific genes was normalized to GAPDH, and data were calculated as fold change relative to the control (chitosan/carbon/cell constructs vs chitosan/cell constructs, n = 4–5 per group and time point).

**Statistics.** Results are expressed as mean ± SD, with n = 3–5 per group and time point. Single factor analysis of variance (ANOVA) was used to determine statistical significance within a data set. If ANOVA detected a significant difference within the data set, Tukey HSD posthoc test was used to determine significant differences between groups. Differences were considered to be significant when p < 0.05.
RESULTS AND DISCUSSION

Scaffold Structure. The porosity, pore interconnectivity, and pore sizes determined by μCT analysis were comparable for chitosan scaffolds and chitosan/carbon scaffolds (Table 1). Chitosan scaffolds had the void volume of 91.5 ± 0.98% and pore interconnectivity of 96.9 ± 1.3%. The pore interconnectivity of the scaffolds was maintained with the incorporation of carbon nanofibers into chitosan, with only a slight (but not statistically significant) decrease in porosity and pore size (Table 1). Overall, no significant differences in scaffold structure were observed with the addition of carbon nanofibers.

Mechanical Properties of Hydrated Scaffolds. The scaffolds were evaluated using dynamic mechanical analysis (DMA)36 in fully hydrated state, over a range of frequencies (0.1, 0.16, 0.25, 0.40, 0.63, 1.0, 1.6, 2.5, 4.0, 6.3, and 10 Hz). The viscoelastic behavior was evaluated by measuring the storage modulus (\(E'\)) and the loss factor (\(tan \delta = E''/E'\), where \(E'\) is the viscous modulus; Figure 2). For both scaffolds, the elastic modulus increased continuously over the tested frequency range, and was at all frequencies higher for the chitosan/carbon scaffolds than pure chitosan scaffolds. At 1 Hz, a slight but significant difference was observed in the elastic moduli of chitosan and chitosan/carbon scaffolds (Table 1), and both values were in the range of those measured for muscle (8–17 kPa)37 and myocardium in healthy rats (18 kPa),38 indicating the ability of the scaffolds to store energy under cyclic load.

The values of the loss factor \(tan \delta\) in Figure 2 indicate that the damping properties of both scaffolds increased with frequency, and were not significantly changed by the incorporation of carbon nanofibers. In both the chitosan and chitosan/carbon scaffolds, the loss factor had values between 0.14 and 0.32, indicative of viscoelastic properties, with the viscous component of the dynamic modulus \(S\) times lower than the elastic modulus. In general, the values of the loss factor indicate that the scaffolds have ability to recover under cyclic loads, and that the relaxation times shortened at higher frequencies, such that the scaffolds dissipated more energy under cyclic loading. Native cardiac tissues also exhibit damping properties under cyclic loading, with hysteresis between loading–unloading stress–strain curves. For example, bovine pericardial tissue dissipates about 20% of the loading energy during loading–unloading, at frequencies of 0.1–10 Hz.39

Scaffold Swelling and Degradation. Material resorption is generally desired, and the degradation products must be nontoxic and readily eliminated from the body. Ideally, the degradation rate of the scaffold should correspond to the regeneration rate of the new tissue. To evaluate degradation, the scaffolds were immersed in PBS solution for 60 days under both static and agitation conditions. Chitosan is a hydrophilic polymer, and the chitosan scaffolds absorbed PBS to ~550% of their weight and maintained this degree of swelling over 60 days of incubation (Figure 3). The lower water uptake of chitosan/carbon scaffolds was proportionate to the fraction of carbon nanofibers, which are hydrophobic, within the hydrophilic chitosan scaffold matrix (Figure 3).

Over 60 days, the structural integrity of chitosan/carbon scaffolds was maintained, without measurable loss of material due to degradation. In contrast, degradation of chitosan scaffolds without carbon was observed in our previous studies in the presence of lysozyme,40 an enzyme ubiquitous in the human body40,41 that degrades chitosan in vitro and in vivo.42,43

In that study, lysozyme was used at concentrations similar to those found in human serum,18 at pH 5.44 We also investigated the in vivo degradation of chitosan scaffolds over 3 months21 and again observed a weight loss as a function of time (3, 6, and 12 weeks).21 Taken together, these data establish the kinetics and mechanisms of chitosan degradation and support the need for future studies evaluating the degradation of chitosan/carbon scaffolds in vivo.

Electrical Conductivity. A comparative analysis of the conductivity of the scaffolds shows that carbon nanofibers strongly improved the electrical conductivity (Table 1). The electrical conductivity of chitosan/carbon scaffolds, measured in a dry state as in previous studies,30,31 was 9 orders of magnitude above that of chitosan scaffolds. Importantly, the electrical conductivity of chitosan/carbon scaffolds is in the same order of magnitude as the conductivity of ventricular muscle, blood, and skeletal muscle (0.03–0.6 S/m).45

After the scaffolds are hydrated, bulk conductivity of the bulk material is largely determined by the conductivity of liquid, so that the hydrated chitosan scaffolds markedly increased their conductivity compared with dry scaffolds (0.03 ± 0.02 S/m vs 7.4 × 10⁻⁹ ± 1.0 × 10⁻⁹ S/m, Table 1). In the hydrated chitosan/carbon scaffolds, the conductivity was determined by both chitosan/carbon and solution (0.04 ± 0.02 S/m, Table 1). However, the microscopic conductivity of the scaffold material remains significantly higher for chitosan/carbon scaffolds. The local changes in conductivity are important as the carbon nanofibers can directly affect the membrane properties of the cardiomyocytes, by altering their resistance or capacitance even without changing the conductivity of the scaffold in its hydrated state.

Metabolic Activity. Cardiomyocytes represent only about 30% of the cells in the adult myocardium and have only a minimal ability to differentiate, proliferate, and self-renew.46 The remaining 70% of cells are endothelial cells and fibroblasts, which have capacity to proliferate. However, cardiomyocytes are the most metabolically active cells in the human body.47 MTS assay involves the biological reduction by viable cells of the tetrazolium compound (MTS). The MTS assay reagent is composed of MTS and the electron coupling agent phenazine methosulfate (PMS). The formazan product of MTS reduction is soluble in tissue culture medium. This reaction only takes place when mitochondrial reductase enzymes are active, and therefore, the conversion can be directly related to the viability of cells in culture.

Figure 3. Swelling properties. Water uptake by the scaffolds (6 mm diameter × 1.5 mm thick cylinders) as a function of immersion time in PBS (pH 7.4, 37 °C). Data are shown as % increase in the scaffold weight, as means ± SD (n = 4 per group and time point).
In chitosan and chitosan/carbon scaffolds, cells were able to reduce the MTS after 7 and 14 days of culture, without significant increases in metabolic rates as a function of time (Figure 4). After 14 days of culture, the metabolic activity of the cells cultured in chitosan/carbon/cell constructs was markedly and significantly higher than in chitosan scaffolds. Importantly, cell viability was maintained throughout the duration of culture. These results can be attributed to the actual differences in cell metabolic rates, as all experiments were conducted using the same cell preparations, and with efficiencies of cell seeding into the scaffolds of approximately 80%.

**Microstructure.** Scaffolds for cardiac applications should have an interconnected structure for cell infiltration, and appropriate porosity and pore dimensions allowing high densities of seeded cells, vascularization, and effective transport of nutrients and oxygen. Myocardial cells have dimensions in the range of 10–100 μm, and the pore sizes in seeded scaffolds ranged from ~120–150 μm, as measured by SEM and histology. Morphological observation by SEM indicated homogeneous dispersion of carbon nanofibers throughout chitosan matrix (Figure 5). SEM images show highly porous scaffolds with pores infiltrated with cells after 7 and 14 days of culture, corroborating the results of μCT that showed high void volumes and high interconnectivity of the pores (Table 1).

**Histomorphology.** Histological cross sections of chitosan and chitosan/carbon scaffolds cultured with cardiomyocytes for 7 and 14 days (Figure 6) were consistent with the porosity, interconnectivity and size of pores measured by μCT. The cellular infiltration throughout the scaffolds was evident (Figure 6). The scaffold pores were filled with cells and well distributed throughout the scaffold volume. No significant differences in cell infiltration were observed between chitosan and chitosan/carbon scaffolds. Histological and SEM evaluations indicate that the cells assume elongated shape and start to align with each other, suggesting the need for more detailed and quantitative assessment in further studies.

**Gene Expression (Real Time-qPCR).** Cardiac gene expression profiles were analyzed for cardiomyocytes cultured in chitosan and chitosan/carbon scaffolds (Table 2), to evaluate the effect of carbon nanofibers on the expression of specific cardiac genes. After 7 days of culture, all genes were overexpressed in cells on chitosan/carbon scaffolds, with the exception of Anf. Specifically, the expression of Tnnc1 and Cx43 increased 2- and 2.6-fold, respectively. The highest gene expression after 7 days of culture was observed for cardiac troponin Tnnc1 (important for contractile function in muscle tissue) and Cx43 (important for conduction of electrical signals).

Functional neonatal cardiomyocytes communicate by electrical impulses through gap junctions. This ability was enhanced...
by the presence of carbon nanofibers in chitosan scaffolds, as evidenced by elevated expression of Cx43 after 7 days (Figure 7). The higher Cx43 level on day 7, without electrical stimulation, suggests that the carbon nanofibers affect physiology independently of possible complementary effects of the external electrical field. Notably, this high expression was maintained and slightly (but not significantly) increased by day 14 of culture (Figure 7, Table 2).

Significant increases were also observed in the expression of Anf, Myh6, Myh7, Tnc1, Gata4, and Atpa2a genes over time (Figure 7). Atrial natriuretic factor (Anf) is a powerful vasodilator, a marker of cardiomyocyte differentiation, and a protein secreted by heart muscle cells was upregulated after 14 days (p < 0.01). Gata4, a survival factor for differentiated cardiomyocytes and an important regulator of postnatal heart function, was also significantly upregulated (p < 0.01) after 14 days (Figure 7). The expression and activity of ATPase 2a has been observed to decrease in cardiomyocytes in a failing heart, and the overexpression Atpa2a could restore the cardiac function in heart failure by improving calcium handling in the cardiomyocytes. Significant increase in the expression of ATP2a2 was observed in chitosan/carbon scaffolds with time in culture (Figure 7), with 4-fold higher expression relatively to control (chitosan scaffolds) after 14 days of culture (Figure 7, Table 2).

Real time qPCR thus confirms that the presence of carbon nanofibers in chitosan scaffolds enhanced the cardiogenic phenotype and expression of cardiac markers after 14 days, without external electrical stimulation. In future studies, these data will be complemented by analysis of cardiac-specific proteins in engineered cardiac constructs. Given that the macroscopic conductivity of the scaffolds in their hydrated state was not significantly different between chitosan/carbon and chitosan scaffolds, the enhanced conductivity at the microscopic scale (at the level of carbon nanofibers) clearly played a role in enhancing gene expression, possibly through microscopic gradients in voltage and currents around the cells.

### CONCLUSIONS

We describe electrically conductive composite scaffolds based on chitosan, designed to have mechanical properties similar to those of cardiac muscle, and to provide local increases (by 9 orders of magnitude) in electrical conductivity by addition of carbon nanofibers. The carbon/chitosan scaffolds supported...
cultivation of cardiac cells and enhanced cardiogenic properties without exogenous electrical stimulation. In the presence of carbon nanofibers, cardiomyocytes were able to adhere and survive for up to 14 days, and elevate their metabolic activity and expression of cardiac genes. Gene expression profiling showed upregulation of Tnn1 and Cx43 in chitosan/carbon/cell constructs after 7 days of culture and all measured cardiogenic genes after 14 days of culture. Looking forward, future studies should focus on spontaneous beating and functionality of cardiac cells in these scaffolds and mapping of the electrical signal transmission.

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Notes
The authors declare no competing financial interest.

 ■ ACKNOWLEDGMENTS
Funding for this work was provided by the “Fundação para a Ciência e Tecnologia” (Postdoctoral Grant SFRH/BPD/66897/2009 to A.M.M. financed by POPH - QREN – Advanced Formation, cofinanced by Social European Fund and National Fund from MCTES) and NIH (Grants EB002520 and HL076485 to G.V.-N.). The authors thank Dr. Arantz Villasante and Dr. Kara Spiller (Columbia University) for their help with qPCR experiments and data analysis, and Prof. Senentxu Lancers-Mendez and Catarina Lopes (University of Minho) for their assistance with the electrical conductivity measurements.

 ■ REFERENCES
(1) Shin, M.; Ishii, O.; Sueda, T.; Vacanti, J. P. Contractile cardiac grafts using a novel nanofibrous mesh. Biomaterials 2004, 25, 3717–3723.

(2) Leor, J.; Landa, N.; Cohen, S. Renovation of the injured heart with myocardial tissue engineering. Expert Rev. Cardiovasc. Ther. 2006, 4, 239–252.

(3) Yang, M. C.; Wang, S. S.; Chou, N. K.; Chi, N. H.; Huang, Y. Y.; Chang, Y. L.; Shieh, M. J.; Chung, T. W. The cardiomyogenic differentiation of rat mesenchymal stem cells on silk fibroin-polysaccharide cardiac patches in vitro. Biomaterials 2009, 30, 3757–3765.

(4) Barsotti, M. C.; Felice, F.; Ballarini, A.; Di Stefano, R. Fibrin as a scaffold for cardiac tissue engineering. Biotechnol. Appl. Biochem. 2011, 58, 301–310.

(5) Zhang, T.; Wan, L. Q.; Xiong, Z.; Marsano, A.; Maidhof, R.; Park, M.; Yan, Y. N. A.; Vunjak-Novakovic, G. Channeled scaffolds for engineering myocardium with mechanical stimulation. J. Tissue Eng. Regener. Med. 2012, 6, 748–756.

(6) Patra, C.; Talukdar, S.; Novoyatleva, T.; Velagala, S. R.; Muhlfeld, C.; Kundu, B.; Kundu, S. C.; Engel, F. B. Silk protein fibron from Antherea mylitta for cardiac tissue engineering. Biomaterials 2012, 33, 2673–2680.

(7) Tian, B. Z.; Liu, J.; Dvir, T.; Jin, L. H.; Tsiu, J. H.; Qing, Q.; Suo, Z. G.; Langer, R.; Kohane, D. S.; Lieber, C. M. Macroporous nanowire nanoelectronic scaffolds for synthetic tissues. Nat. Mater. 2012, 11, 986–994.

(8) Zorlutuna, P.; Elsheikh, A.; Hasirci, V. Nanopatterning of collagen scaffolds improve the mechanical properties of tissue engineered vascular grafts. Biomacromolecules 2009, 10, 814–821.

(9) Radisic, M.; Park, H.; Shing, H.; Consi, T.; Schoen, F. J.; Langer, R.; Freed, L. E.; Vunjak-Novakovic, G. Functional assembly of engineered myocardium by electrical stimulation of cardiac myocytes cultured on scaffolds. Proc. Natl. Acad. Sci. U.S.A. 2004, 101, 18129–18134.

(10) Tandon, N.; Marsano, A.; Maidhof, R.; Wan, L.; Park, H.; Vunjak-Novakovic, G. Optimization of electrical stimulation parameters for cardiac tissue engineering. J. Tissue Eng. Regener. Med. 2011, 5, E115–E125.

(11) Dvir, T.; Timko, B. P.; Brigham, M. D.; Naik, S. R.; Karajangui, S. S.; Levy, O.; Jin, H. W.; Parker, K. K.; Langer, R.; Kohane, D. S. Nanowired three-dimensional cardiac patches. Nat. Nanotechnol. 2011, 6, 720–725.

(12) Maidhof, R.; Tandon, N.; Lee, E. J.; Luo, J. W.; Duan, Y.; Yeager, K.; Konofagou, E.; Vunjak-Novakovic, G. Biomimetic perfusion and electrical stimulation applied in concert improved the assembly of engineered cardiac tissue. J. Tissue Eng. Regener. Med. 2012, 6, e12–e23.

(13) Fernandes, E. G. R.; Zaculotto, V.; De Queiroz, A. A. A. Electropinning of hyperbranched poly-l-lysine/polyaniline nanofibers for application in cardiac tissue engineering. J. Macromol. Sci., Part A 2010, 47, 1203–1207.

(14) Stout, D. A.; Basu, B.; Webster, T. J. Poly(lactic-co-glycolic acid): Carbon nanofiber composites for myocardial tissue engineering applications. Acta Biomater. 2011, 7, 3101–3112.

(15) You, J.; O.; Rafat, M.; Ye, G. J. C.; Auguste, D. T. Nanoengineering the heart: conductive scaffolds enhance Connexin 43 expression. Nano Lett. 2011, 11, 3643–3648.

(16) Borriello, A.; Guarriso, V.; Schiavi, L.; Alvarez-Perez, M. A.; Ambrosio, L. Optimizing PANi doped electroactive substrates as patches for the regeneration of cardiac muscle. J. Mater. Sci.: Mater. Med. 2011, 22, 1053–1062.

(17) Stout, D. A.; Yoo, J.; Santiago-Miranda, A. N.; Webster, T. J. Mechanisms of greater cardiomyocyte functions on conductive nanoengineered composites for cardiovascular applications. Int. J. Nanomed. 2012, 7, 5653–5669.

(18) Martins, A. M.; Santos, M. L.; Azevedo, H. S.; Malafaya, P. B.; Reis, R. L. Natural origin scaffolds with in situ pore forming capability for bone tissue engineering applications. Acta Biomater. 2008, 4, 1637–1645.

(19) Martins, A. M.; Pham, Q. P.; Malafaya, P. B.; Raphael, R. M.; Kasper, F. K.; Reis, R. L.; Mikos, A. G. “Smart” and stimulus responsive chitosan-based scaffolds/cells for bone tissue engineering:

Figure 7. Gene expression. Data are shown for real time-qPCR analysis of cardiomyocytes cultured on chitosan/carbon scaffolds for 7 and 14 days vs control samples (chitosan scaffolds cultured using the same conditions). Data are shown as fold change in gene expression relatively to control (chitosan/carbon/cell constructs vs chitosan/cell constructs) and are expressed as means ± SD (n = 4–5 per group and time point). (***) Indicates a significant difference (p < 0.01) and (*) indicates a significant difference (p < 0.05) for the same gene as a function of time.
Influence of lysozyme upon scaffold degradation and osteogenic differentiation of cultured marrow stromal cells induced by cap coatings. *Tissue Eng., Part A* **2008**, *14*, 795–795.

(20) Martins, A. M.; Alves, C. M.; Kasper, F. K.; Mikos, A. G.; Reis, R. L. Responsive and in situ-forming chitosan scaffolds for bone tissue engineering applications: an overview of the last decade. *J. Mater. Chem.*** **2010**, *20*, 1638–1645.

(21) Martins, A. M.; Kersler, J. D.; Costa-Pinto, A. R.; Malafaya, P. B.; Fernandes, E. M.; Neves, N. M.; Alves, C. M.; Mikos, A. G.; Kasper, F. K.; Reis, R. L. Gradual pore formation in natural origin scaffolds throughout subcutaneous implantation. *J. Biomed. Mater. Res., A* **2012**, *100*, 599–612.

(22) Madhally, S. V.; Matthew, H. W. Porous chitosan scaffolds for tissue engineering. *Biomaterials*** **1999**, *20*, 1133–1142.

(23) Ueno, H.; Mori, T.; Fujinaga, T. Topical formulations and wound healing applications of chitosan. *Adv. Drug Delivery Rev.* **2001**, *52*, 105–115.

(24) Hoekstra, A.; Struszczyk, H.; Kivela, O. Percutaneous mechanical properties of chitosan/carbon nanotubes composites. *Langmuir*** **2008**, *24*, 1473–1477.

(25) Kasper, F. K.; Reis, R. L. Gradual pore formation in natural origin chitosan scaffolds. *Biomaterials*** **2002**, *23*, 6127–6132.

(26) Wang, H. B.; Zhang, X. L.; Li, Y. M.; Ma, Y. T.; Zhang, Y.; Liu, Z. G.; Zhou, J.; Lin, Q. X.; Wang, Y. M.; Duan, C. M.; Wang, C. Y. Functional improvement of infiltrated heart by co-injection of embryonic stem cells with temperature-responsive chitosan hydrogel. *Tissue Eng., Part A* **2009**, *15*, 1437–1447.

(27) Lu, W. N.; Lu, S. H.; Wang, H. B.; Li, D. X.; Duan, C. M.; Liu, Z. Q.; Hao, T.; He, W. J.; Xu, B.; Fu, Q.; Song, Y. C.; Xie, X. H.; Wang, C. Y. Functional improvement of infiltrated heart by co-injection of basic fibroblast growth factor with temperature-responsive Chitosan hydrogel. *J. Heart Lung Transpl.* **2010**, *29*, 881–887.

(28) Liu, Z. Q.; Wang, H. B.; Wang, Y.; Lin, Q. X.; Yao, A. N.; Cao, F.; Li, D. X.; Zhou, J.; Duan, C. M.; Du, Z. Y.; Wang, Y. M.; Wang, C. Y. The influence of chitosan hydrogel on stem cell engraftment, survival and homing in the ischemic myocardial microenvironment. *Biomaterials*** **2012**, *33*, 3093–3106.

(37) Wang, S. F.; Shen, L.; Zhang, W. D.; Tong, Y. J. Preparation and mechanical properties of chitosan/carbon nanotubes composites. *Biomacromolecules*** **2005**, *6*, 3067–3072.

(38) Lau, C.; Cooney, M. J.; Atanassov, P. Conductive macroporous composite chitosan-carbon nanotube scaffolds. *Langmuir*** **2008**, *24*, 7004–7010.

(39) Livak, K. J.; Schmittgen, T. D. Analysis of relative gene expression data using real-time quantitative PCR and the 2(T) method. *Methods*** **2001**, *25*, 402–408.

(40) Mano, J. C.; Reis, R. L.; Cunha, A. M. Dynamic Mechanical Analysis in Polymers for Biomedical Applications. In *Polymer Based Systems on Tissue Engineering, Replacement and Regeneration*; Reis, R. L., Cohn, D. Eds.; Dordrecht: Kluwer, 2002; p 139–164.

(41) Engler, A. J.; Sen, S.; Sweeney, H. L.; Discher, D. E. Matrix elasticity directly sets cell lineage specification. *Cell*** **2006**, *126*, 677–689.

(42) Berry, M. F.; Engler, A. J.; Woo, Y. J.; Piroli, T. J.; Bish, L. T.; Jayasankar, V.; Morine, K. J.; Gardner, T. J.; Discher, D. E.; Sweeney, H. L. Mesenchymal stem cell injection after myocardial infarction improves myocardial compliance. *Am. J. Physiol.* **2006**, *290*, H2196–H2203.
(59) Kodama, H.; Inoue, T.; Watanabe, R.; Yasuoka, H.; Kawakami, Y.; Ogawa, S.; Ikeda, Y.; Mikoshiba, K.; Kuwana, M. Cardiomyogenic potential of mesenchymal progenitors derived from human circulating CD14(+) monocytes. Stem Cells Dev. 2005, 14, 676–686.

(60) Oka, T.; Maillet, M.; Watt, A. J.; Schwartz, R. J.; Aronow, B. J.; Duncan, S. A.; Molkentin, J. D. Cardiac-specific deletion of Gata4 reveals its requirement for hypertrophy, compensation, and myocyte viability. Circ. Res. 2006, 98, 837–845.

(61) Dang, X. T.; Doble, B. W.; Kardami, E. The carboxy-tail of connexin-43 localizes to the nucleus and inhibits cell growth. Mol. Cell. Biochem. 2003, 242, 35–38.

(62) Boengler, K.; Heusch, G.; Schulz, R. Connexin 43 and ischemic preconditioning: effects of age and disease. Exp. Gerontol. 2006, 41, 485–488.

(63) Kawase, Y.; Ly, H. Q.; Prounier, F.; Lebeche, D.; Shi, Y. F.; Jin, H. W.; Hadri, L.; Yoneyama, R.; Hoshino, K.; Takewa, Y.; Sakata, S.; Peluso, R.; Zsebo, K.; Gwathmey, J. K.; Tardif, J. C.; Tanguay, J. F.; Hajar, R. J. Reversal of cardiac dysfunction after long-term expression of SERCA2a by gene transfer in a pre-clinical model of heart failure. J. Am. Coll. Cardiol. 2008, 51, 1112–1119.