Singh, N., Koziol, K. K., Chen, J., Patil, A. J., Gilman, J., Truelove, P., ... Rahatekar, S. (2013). Ionic liquids-based processing of electrically conducting chitin nanocomposite scaffolds for stem cell growth. Green Chemistry, 15(5), 1192 - 1202. 10.1039/C3GC37087A

Green Chemistry

Accepted Manuscript

This is an *Accepted Manuscript*, which has been through the RSC Publishing peer review process and has been accepted for publication.

Accepted Manuscripts are published online shortly after acceptance, which is prior to technical editing, formatting and proof reading. This free service from RSC Publishing allows authors to make their results available to the community, in citable form, before publication of the edited article. This *Accepted Manuscript* will be replaced by the edited and formatted *Advance Article* as soon as this is available.

To cite this manuscript please use its permanent Digital Object Identifier (DOI®), which is identical for all formats of publication.

More information about *Accepted Manuscripts* can be found in the **Information for Authors**.

Please note that technical editing may introduce minor changes to the text and/or graphics contained in the manuscript submitted by the author(s) which may alter content, and that the standard **Terms & Conditions** and the **ethical guidelines** that apply to the journal are still applicable. In no event shall the RSC be held responsible for any errors or omissions in these *Accepted Manuscript* manuscripts or any consequences arising from the use of any information contained in them.

Ionic Liquids-Based Processing of Electrically Conducting Chitin Nanocomposite Scaffolds for Stem Cell Growth

Nandita Singh[1], Krzysztof K.K Koziol[2], Jinhu Chen[2], Avinash J. Patil[3], Jeffrey W Gilman[4†], Paul Trulove[5], Wael Kafienah[1*], and Sameer S. Rahatekar[6*]

1) School of Cellular & Molecular Medicine, University of Bristol, Bristol BS8 1TD, UK

2) Department of Materials Science and Metallurgy, University of Cambridge, Cambridge CB2 3QZ, UK

3) Centre for Organized Matter Chemistry, School of Chemistry, University of Bristol, Bristol BS8 1TS, UK

4) Sustainable Polymers Group, Materials Science and Engineering Division, National Institute of Standards and Technology, Gaithersburg, MD 20899, USA

5) Department of Chemistry, United States Naval Academy, Annapolis, MD 21402, USA

6) Advanced Composites Centre for Innovation and Science (ACCIS), Aerospace Engineering, University of Bristol, Bristol BS8 1TR, UK

***Corresponding Authors:**

Dr Sameer S Rahatekar

Advanced Composites Centre for Innovation and Science (ACCIS)
Aerospace Engineering, University of Bristol, Bristol BS8 1TR, UK
Tel: +44 117331 5330; Fax: +44 1173315330

Email:Sameer.Rahatekar@bristol.ac.uk

Dr Wael Kafienah
School of Cellular and Molecular Medicine, University of Bristol, Bristol BS8 1TD, UK
Tel +44 117 33 12096; fax: +44 (0)117 33 12091

Email: w.z.kafienah@bristol.ac.uk

Abstract

In the present study, we have successfully combined the biocompatible properties of chitin with the high electrical conductivity of carbon nanotubes (CNTs) by mixing them using an imidazolium-based ionic liquid as a common solvent/dispersion medium. The resulting nanocomposites demonstrated uniform distribution of CNTs, as shown by scanning electron microscopy (SEM) and optical microscopy. Fourier transform infrared spectroscopy (FTIR) and X-ray diffraction confirmed the α-crystal structure of chitin in the regenerated chitin nanocomposite scaffolds. Increased CNT concentration in the chitin matrix resulted in higher conductivity of the scaffolds. Human mesenchymal stem cells adhered to, and proliferated on, chitin/CNT nanocomposites with different ratios. Cell growth in the first 3 days was similar on all composites at a range of (0.01 to 0.07) mass fraction of CNT. However, composites at 0.1 mass fraction of CNT showed reduced cell attachment. There was a significant increase in cell proliferation using 0.07 mass fraction CNT composites suggesting a stem cell enhancing function for CNTs at this concentration. In conclusion, ionic liquid allowed the uniform dispersion of CNTs and dissolution of chitin to create a biocompatible, electrically conducting scaffold permissive for mesenchymal stem cell function. This method will enable the fabrication of chitin-based advanced multifunctional biocompatible scaffolds where electrical conduction is critical for tissue function.

Keywords: Chitin, carbon nanotubes, ionic liquids, stem cells, biomaterials.

1. Introduction

Chitin, which is derived from sea creatures and insects[1], is the second most abundant and renewable polymer in the world after cellulose[2]. Despite its abundance, excellent mechanical properties and biocompatibility, chitin is not used extensively due to the lack of a benign solvent for effective dissolution and processing into a final product. Traditionally, harsh chemicals like lithium bromide[3], formic acid and hexafluoroisopropanol[4] are used for processing chitin. In the last decade, ionic liquids have emerged as a new generation of benign solvents for dissolving cellulose, chitin and

other natural polymers[5-8]. Recently, a small number of researchers have carried out chitin processing using ionic liquids to prepare films, fibres, gels and foams[9-13]. However the resulting regenerated chitin was electrically non-conductive and hence could only be used for monofunctional tasks. The goal of the current work is to manufacture electrically conductive and biocompatible chitin and multiwall carbon nanotube (MWNT) composite scaffolds, which can support cell growth and electrical stimulation. Such a scaffold would be of value in regenerative medicine for promoting the differentiation of stem cells into a specific lineage [14,15], increasing the functionality of cardiomyocytes [16] and neurons [17], and increasing the cell density of chondrocytes[18]. Composites of chitin/MWNT will have the advantage of chitin's natural origin[19], exceptionally low immunogenicity, antimicrobial activity and biocompatibility [20] and MWNTs excellent electrical conductivity and high aspect ratio. Importantly, the blending of chitin and MWNTs should minimise or even avoid the toxic effects of nanotubes reported elsewhere[21]. A soft and biocompatible chitin-based electrically conductive scaffold, as proposed in the current study, is likely to be more effective in the electrical stimulation of cells than traditionally-used metal-based conducting scaffolds/implantable electrodes that demonstrate a limited lifespan *in vivo* due to an elastic mismatch between the metal and surrounding tissues[22].

To manufacture chitin/MWNT composites we have used ionic liquid as a common platform for dissolution of chitin, as well as exfoliation and good dispersion of MWNTs. Uniform dispersion of MWNTs in most common solvents and polymers present a major challenge in the preparation of polymer nanocomposites [23]. In the present work we have developed a non-covalent functionalisation method of carbon nanotubes that can achieve homogeneous dispersion of MWNTs in ionic liquid, which can subsequently be used as a common solvent to dissolve chitin and fabricate well-dispersed electrically conductive chitin/MWNT composite films. The biocompatibility of these multifunctional electrically conductive films was evaluated by testing the viability and proliferation of mesenchymal stem cells (MSCs) on the chitin/MWNT composite films.

2. Materials and Methods

2.1 Preparation of the chitin nanotube films

Chitin (MW 100,000) was purchased from Heppe Medical Chitosan GmBH (Germany). The ionic liquid, 1-ethyl-3-methylimidazolium acetate (EMI Ac) was used as a solvent for chitin and was purchased from Sigma Aldrich. All the tissue culture chemicals were purchased from Sigma unless otherwise stated. The two-dimensional membranes of chitin were produced using the following procedure: 0.015 weight fraction of chitin was dissolved in 5 g of EMI Ac in a glass vial accompanied by constant heating and stirring for 2 h at 130 °C. Multiwall carbon nanotubes (MWNTs) coated with carboxymethyl cellulose were used to prepare chitin and MWNT composite films. To prepare the carboxymethyl cellulose coated MWNTs, the chemical vapour deposition (CVD)-grown highly-aligned MWNTs (the process for synthesis of which has been described in our previous work [24]) were mixed with carboxymethyl cellulose and distilled water solution. The MWNT and carboxymethyl cellulose solution was sonicated for 30 minutes to achieve good dispersion of the MWNTs in water, as shown in Figure 1A. It has been shown that carboxymethyl cellulose is an excellent surfactant allowing uniform dispersion of MWNTs in aqueous medium[25-28]. The dispersed MWNTs were dried to remove water, resulting in MWNTs coated with carboxymethyl cellulose. The carboxymethyl cellulose coated MWNTs were then dispersed in EMI Ac by heating them at 60 °C for 12 h. We found that carboxymethyl cellulose could be solvated in EMI Ac; hence the carboxymethyl-coated MWNTs (referred as coated MWNTs henceforth) could be easily dispersed in EMI Ac. Chitin was added to the coated MWNT suspension in EMI Ac so as to achieve 0.01, 0.03, 0.07 and 0.1 mass fraction of MWNTs with respect to the amount of dissolved chitin. The solution was poured into a glass petridish and allowed to cool for 3 h. The cooled solution was coagulated by adding ethanol to the petri dish. The ethanol selectively dissolves the EMI Ac and coagulates the chitin. The coagulated films were soaked in distilled water for 2 d to remove traces of EMI Ac[29] then dried at room temperature. Figure 1A shows a diagrammatic representation of the entire process for the preparation of membranes. Figure 1B shows optical microscope images of the final chitin/MWNT composites films with 0.01, 0.03, 0.07 and 0.1 weight fractions of MWNTs.

2.2 Characterisation of Chitin/MWNT Composite Films

Scanning electron microscopy: The cross section of chitin/MWNT films was analysed using scanning electron microscopy (SEM) imaging with a field emission gun scanning electron microscope (JEOL JSM-6340 FEGSEM) with an accelerating voltage of 15.0 kV and working distances between 15 mm and 6 mm. Thin 2D films of chitin/MWNT composites were fixed to an aluminium stub with a carbon pad. In order to avoid surface charging, a thin film of gold was sputtered onto the samples with an EMITECH sputter coater.

X-ray diffraction: X-ray diffraction was used to identify the presence and type of crystalline structures in the samples. We used the Bruker Nanostar diffractometer with 40 kV/40 mA X-ray gun settings and 2D Hi star detector. The 2D transmission mode X-ray diffraction studies were performed using Cu Kα radiation (λ=0.154 nm) as a source of X-rays. The 2D diffraction patterns were analyzed and converted into a 1D plot using the radial integration function.

FTIR analysis: The molecular structure of chitin/MWNT films was assessed using Fourier transform infrared spectroscopy (FTIR) analysis. The analysis was carried out in transmission mode using a Spectrum 100 FTIR spectrometer (PerkinElmer, Waltham, MA, USA).

Conductivity measurement: The electrical conductivity testing was carried out with 2-point probe conductivity setup (Keithley 2000 multimer). A thin strip of the film, 5 mm by 20 mm, was cut and placed on the glass slide held in place by tape. The film's contact points with the electrodes were coated with silver paint to reduce the contact resistance. The resistance of the films over a distance of 2 mm was measured. The electrical conductivity of the chitin and carbon nanotube composite films was calculated using the equation:

$$\sigma = L / (R \times A)$$

R is the resistance measured over length L, and A is the cross-sectional area of the sample.

2.3 Cell culture

Mesenchymal stem cells obtained from human bone marrow were used for this study. Bone marrow plugs were collected from the femoral head of patients undergoing total hip arthroplasty. All patients provided informed consent and the study was carried out according to local ethical guidelines. Cells were suspended in stem cell expansion medium consisting of low glucose Dulbecco's Modified Eagles Medium supplemented with 0.1 volume fractions of Foetal Bovine Serum (FBS, Thermo Scientific Hyclone, Loughborough, UK), 0.01 volume fraction Glutamax (Sigma, Poole, UK) and 0.1 volume fractions of Penicillin G (10,000 units/ml)/Streptomycin (10,000 mg/ml) antibiotic mixture (P/S; Sigma). The serum batch was selected to promote the growth and differentiation of mesenchymal stem cells[30]. The medium was supplemented with 2 ng/ml FGF-2 (PeproTech) to enhance MSC proliferation and differentiation [31]. The cell suspension was separated from any bone in the sample by repeated washing with media. The cells were centrifuged at 500 *g* for 5 minutes and the supernatant/fat removed. The resulting cell pellet was resuspended in medium and plated at a seeding density between 1.5 $x10^5$ and 2.0 $x10^5$ nucleated cells per cm^2. These flasks were incubated at 37 °C in a humidified atmosphere of 0.05 volume fractions of CO_2 and 0.95 volume fractions of air. All experiments were done with passage two cells.

Dry membranes were cut with a biopsy punch to produce 8 mm diameter discs. These were placed in a 24-well tissue culture plate. The membranes were disinfected with 0.7 volume fractions of ethanol for 30 minutes and washed a few times with sterile PBS. The membranes were coated with fibronectin (100 µg/ml) for 5 h at 37 °C, washed with PBS and transferred to ultra-low attachment plates to dry overnight.

Cells were loaded on the fibronectin-coated scaffolds at a density of $2.8x10^4$ cells per cm^2. The seeded cells were cultured in expansion medium with FGF-2 at 2 ng/ml. Cells seeded on plastic as positive controls were maintained in the same medium.

2.4 Cell adhesion and viability assay

Live monitoring of cell adhesion and viability was conducted using the LIVE⁄DEAD Viability/Cytotoxicity Kit for mammalian cells (Invitrogen, Paisley, UK) as per manufacturer's instructions. Cell-loaded scaffolds were incubated for 3 d and 14 d at 37 °C in a humidified atmosphere of 0.05 volume fractions of CO_2 and 0.95 volume fractions of air. The constructs were washed with PBS and incubated with a kit reagent that stains live cells with green fluorescent dye (calcein AM, emission 488 nm) and dead cells with red fluorescent dye (ethidium homodimer-1, emission 568 nm). Briefly, calcein-AM is membrane-permeable but, once inside the cell, the AM group is cleaved by cellular esterases trapping the calcein in the cell. A loss of cell membrane integrity allows the cleaved calcein to leak from the cell into the surrounding medium leaving only the intact viable cells to fluoresce green. Dead cells, which retain ethidium homodimer-1 through damaged membranes, produce red fluorescence. Negative controls consisting of cells killed with methanol and positive controls consisting of cells grown on plastic tissue culture plates were run with each set of experiments. The plates were viewed under a digital fluorescence microscope system (Leica DMIRB inverted microscope, Houston, TX, USA).

2.5 Cell proliferation assay

Cell proliferation was evaluated using the CellTiter 96 AQueous One Solution MTS Cell Proliferation Assay (Promega) according to the manufacturer's instructions. The assay is based on the reduction of a tetrazolium compound [3-(4, 5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium, inner salt (MTS)] to a coloured formazan product, which is measured spectrophotometrically. The MTS assay was done in triplicate over a time course of 3 d and 14 d. The cells were loaded on the fibronectin-coated scaffolds and dried overnight with a density of $28x10^3$ cells per cm^2. Cells cultured on plastic in a 96-well plate were used to construct the standard curve. The required volume of MTS solution was incubated with the constructs for 2 h at 37 °C in a 0.05 volume fraction CO_2 incubator. The reaction was stopped by adding 25 µL of 0.1 mass by volume fraction SDS. 100 µl of MTS (purple-coloured) solution was transferred to a fresh 96-well plate for reading

from all the samples. The plates were read with a 96-well microplate reader at 490 nm. Each data point was obtained in triplicate.

2.6 Statistical analysis

Comparisons between groups were performed using unpaired Student's *t*-tests (two groups) or one-way analysis of variance (more than two groups). A p value < 0.05 was considered statistically significant. All statistical analyses were performed using Predictive Analytics SoftWare, PASW (v18, IBM, Chicago).

3. Results

3.1 Dispersion of MWNTs

The optical microscopy images of MWNTs dispersed in 1-ethyl-3-methylimidazolium acetate (EMI Ac) was carried out to study whether the carboxymethyl cellulose coated MWNTs show improved dispersion compared to unmodified MWNTs. Figure 2 shows the qualitative comparison of the level of dispersion achieved by MWNTs dispersed in EMI Ac. As seen in Figure 2 a, the uncoated MWNTs show large aggregated clusters whereas the carboxymethyl cellulose coated MWNTs show much more uniform dispersion and very small aggregated clusters (Figure 2 b). Since carboxymethyl cellulose is noncovalently functionalised on the MWNT surface, the resulting polymer-wrapped MWNT can be readily suspended in EMI Ac. The suspension of carboxymethyl cellulose coated MWNTs in EMI Ac was stable for several months. By contrast, the non-functionalised MWNTs did not have this dissolution effect and hence show large aggregated clusters. The acetate ion in EMI Ac can potentially form hydrogen bonding with the carboxymethyl cellulose. This enables facile mixing of the coated MWNTs in EMI Ac and, importantly, maintains the dispersion of the MWNTs once the chitin dissolves.

3.2 Morphological and molecular characterisation of chitin/MWNT films

Scanning electron microscopy (SEM) analysis was carried out to investigate the surface structure and morphology of films prepared from chitin/nanotube composites with increasing CNT concentrations. Figure 3 shows SEM of MWNTs dispersed in chitin films at low (Fig 3 a, c, e, and g) and high (Fig 3 b, d, f and h) magnification. A cross section of chitin/MWNT composite films reveals that MWNTs were uniformly dispersed within the chitin matrix with no evidence of aggregation even when MWNT concentration was increased. This clearly indicated that the wrapping of MWNTs using carboxymethyl cellulose facilitates a good degree of dispersion in the chitin matrix.

In order to understand the molecular crystal structure of regenerated chitin/MWNT composites, physio-chemical analysis using X-ray diffraction and infrared spectroscopy (FTIR) were carried out. The wide angle X-ray diffraction data of 0.01 mass fraction (lowest concentration) and 0.1 mass fraction (highest concentration) of chitin/MWNT films (Figure 4) shows peaks at 2 θ equal to 9.3, 12.5 and 19.3°. The presence of these peaks confirms the α-chitin structure of the regenerated chitin films [12, 32-34] with an anti-parallel chain arrangement as reported in previous studies [35]. The presence of carbon nanotubes can be confirmed by the peak at 26.3° which arises from the multiwall carbon nanotube spacing corresponding to 0.34 nm.

The Fourier transform infrared (FTIR) analysis was carried out to confirm the molecular structure of regenerated chitin and MWNT composite films. Figure 5 shows the FTIR spectra of chitin film reinforced with 0.01 mass fraction (lowest concentration) and 0.1 mass fraction (highest concentration) MWNTs in chitin films. Both these films show a strong peak at 3444 cm^{-1} which corresponds to the O-H vibration [33], and a peak at 3265 cm^{-1} which corresponds to the N-H vibration [33]. The amide-I peak for all the film samples splits into two peaks, one at 1660 cm^{-1} and another at 1624 cm^{-1}, due to the intramolecular hydrogen bonding between C-O and $HOCH_2$ [36-39] present in α-chitin structure. Such splitting was not observed in the β-chitin structure [33]. The amide II peak can be observed at 1554 cm^{-1} and the C-O-C ring vibration peak can be seen at 1155 cm^{-1}. The FTIR analysis, as well as the X-

ray diffraction analysis, confirms the presence of an α-chitin structure in the regenerated chitin and MWNT films.

3.3 Electrical Conductivity of Chitin/MWNT Composite Films

The electrical conductivity of chitin and MWNT composite film was measured as described above. Figure 6 shows the electrical conductivity of chitin and MWNT composite films as a function of MWNT mass fraction. The electrical conductivity of 0.07 mass fraction and 0.1 mass fraction chitin/MWNT films was found to be significantly higher (10 S/m) than that for 0.01 and 0.03 mass fraction chitin and carbon nanotube composites (0.03 S/m). These results indicate that MWNT/chitin composite films have a range of electro-conductive capacities that correlates directly with MWNT mass fraction. The resistance of the neat chitin film was found to be more than 100MΩ which was for all practical purposes was a non- conducting film. This observation is consistent with the very low value of conductivity of neat chitin reported by previous researchers[40].

3.4 Stem cell viability assay of chitin/MWNT composite films

Having measured the electrical conductivity of chitin/MWNT composites films, the next step was to assess the biocompatibility of these films to determine how well mesenchymal stem cells (MSCs) can survive on the surface of these films. The MSC viability on all the chitin/MWNT films was tested using the LIVE/DEAD® viability assay as described above, where green fluorescence indicates live cells and red fluorescence shows dead cells. As seen in Figure 7, a large proportion of live cell attachment was observed after 3 d and 14 d with a negligible number of dead cells on all chitin and MWNT composite films. However, since the film transparency is significantly reduced for 0.07 and 0.1 mass fraction chitin/MWNT films (Figure 1 B), the MSCs do not appear as fluorescently bright as with the more transparent films. The negative control sample for the assay was the chitin/MWNT film without MSCs. These films had no background green or red fluorescence (data not shown). Images for positive controls using cells seeded on plastic (Figure 7I) and negative controls using cells killed by methanol (Figure 7 J) are shown for comparison.

3.5 MTS assay

The LIVE/DEAD® assay gives a qualitative indication of MSC survival without any quantitative measure of the number of MSCs present and their proliferation rate. To determine the growth rate and number of MSCs present on the chitin/MWNT films at various MWNTs concentrations, MTS assay was performed. Tissue culture plastic was treated as a positive control for this analysis. Figure 8 shows the number of MSCs on a given set of membranes after 3 d and 14 d. All chitin/MWNT composite films from (0.01 to 0.1) mass fraction showed MSC attachment. We found that the MSC attachment level at 0.07 mass fraction was significantly higher compared to all the other membranes at day 14. It is interesting to note that the concentration of carbon nanotubes beyond 0.07 mass fraction of MWNTs in the chitin matrix is not ideal for good attachment and proliferation of MSCs. Cell attachment at 14 d was significantly better in both the 0.07 and 0.1 mass fraction membranes (p = 0.042 and 0.019 respectively, *vs.* 3 d).

4. Discussion

In this study, we have demonstrated that ionic liquid can be used as a common solvent for the benign dissolution and processing of chitin, as well as for the uniform dispersion of non-covalently functionalised MWNTs, in the manufacture of electrically conductive chitin/MWNT composite films. We have further shown that bone marrow-derived MSCs can successfully grow and proliferate on the electrically conductive chitin/MWNT composite films.

Traditionally, chitin has been processed using highly aggressive solvents such as hexafluoroisopropanol (HFIP) or formic acid [41]. Although ionic liquids have shown certain toxicological effects depending on the nature of cation, anion and alkyl chain length attached to cation[42, 43] in our opinion the advantage of imidazolium-based ionic liquids in the processing of chitin is the improved operational safety compared to use of sodium hydroxide / carbon di-sulphide / sulphuric acid based solvents (viscous process for dissolving cellulose and chitin), or formic acid and hexafluoro isopropanol, which are highly volatile and can cause severe skin burns and eye damage (based on the Materials Safety Data Sheet for formic acid and hexafluro isopropanol). The imidazolium-based ionic liquids have been shown to have a very low vapour pressure[44, 45] low flammability [46]and lower risk when in direct contact

with skin (based on the Materials Safety Data Sheet for EMI Ac). EMI Ac was shown to have negligible toxicological effect on growth of *Clostridium* sp. bacteria below q concentration of 2.5 g/l[47]. The ease of handling EMI Ac when processing chitin reduces the health hazard and the need for investment in appropriate safety equipment (required for handling more aggressive solvents such as hexafluoroisopropanol and formic acid). Hence we believe that large-scale processing of chitin using EMI Ac will be safer and more economical. To study the long-term ecological impact of EMI Ac, detailed toxicological studies of its influence on aqueous organisms and its toxicological effects during biodegradation are necessary.

In this study, we have shown that EMI Ac can also be effectively used as a common solvent for achieving uniform dispersion of carbon nanotubes as well as for processing natural polymer nanocomposites. Given the ability of ionic liquids to dissolve a wide range of natural polymers, the method proposed here will allow researchers to use ionic liquids as a common platform to disperse nanoparticles and then dissolve a range of natural, sustainable polymers to prepare nanocomposites in one step[8, 10]. Such a technique will lead to benign and more efficient ways to prepare natural polymer-based, electrically conductive nanocomposites from carbon nanotubes that can be used as multifunctional materials.

The electrical conductivity of the 0.01 and 0.03 mass fraction MWNT composites was found to be low, whereas the 0.07 and 0.1 mass fraction MWNT-based composites was significantly higher. These results indicate that the percolation transition for chitin/MWNT composite films occurs between 0.03 and 0.07 mass fractions of MWNT concentration.

The live and dead assay confirmed the viability of cells on all the chitin/MWNT composite film scaffolds up to 14 d. The qualitative assay revealed an increased cell binding capacity with the 0.07 mass fraction MWNT composites. The poor binding of cells on the 0.1 mass fraction chitin/MWNT scaffold as compared to the 0.07 mass fraction is likely to be due to increased hydrophobicity with higher nanotube concentrations. Similar behaviour was reported by Koga *et al* while studying the interaction between primary hepatocytes and carbon nanotubes (CNTs)[48]. They

reported that there was a weak interaction between the two depending on the density of CNTs used and observed that CNTs promote spheroid formation, which increases with high density. Thus 0.07 mass fraction chitin/MWNT nanocomposites remain the most suitable scaffolds for attachment and proliferation of adult MSCs as well as for achieving high electrical conductivity.

Previous studies have used neat carbon nanotubes films/fibres[49-52] and carbon nanotube-based composites with synthetic polymers such as polycarbonate urethane and polylactic acid (PLA) as electrically conductive substrates for cell growth[53]. However, the degraded products from polyurethane or polylactic acid-based scaffolds are known to cause inflammation[54-56]. The chitin/MWNT carbon nanotube composite can overcome this limitation due to the natural origin of chitin, its exceptionally low immunogenicity, antimicrobial activity and biocompatibility. The electrical stimulation of polycarbonate urethane/carbon nanotube composite-based scaffolds showed improved cell density of chondrocytes[18] and PLA/carbon nanotube composite scaffolds showed improved alignment of adipose-derived stem cells[50]. The electrical conductivity of our chitin/MWNT composites was found to be higher than the PLA/carbon nanotube composite scaffolds, as well as for the polycarbonate urethane/carbon nanotube composites as reported by previous researchers for electrical stimulation of cells. This observation suggests that our chitin/MWNT composites have sufficiently high conductivity for the electrical stimulation in tissues.

Currently, the lifetime of prototype electrodes based on metal or silicon fibre is restricted at 3 to 6 months[22]. This is due to a mismatch in the elastic/mechanical properties of the stiff metal/silicon-based electrodes and the surrounding soft tissues, which causes premature loss of electrode function. The soft, electrically conductive and biocompatible chitin/MWNT scaffolds presented in the current work offer a method to overcome this limitation and design a new generation of implantable electrodes.

Carbon nanotubes have excellent electrical conductivity and a high aspect ratio, secondary to which they can achieve electrical percolation in composite materials at a very low weight fraction[57-59]. However, a certain class of carbon nanotubes have been shown to have a toxicological effect on biological cell growth[60, 61]. On the other hand, some researchers have also shown an enhanced function of neurons[62, 63] and

increased density of chondrocytes[18] when they were grown on electrically conductive neat carbon nanotube films and composite scaffolds. To minimise the toxicological effect of carbon nanotubes we have used at least a 0.9 weight fraction of biocompatible chitin matrix with a small fraction of MWNTs. We were able to achieve soft biocompatible as well as electrically conductive scaffolds by using a small weight fraction of carbon nanotubes and a biocompatible chitin polymer. As mentioned in the previous discussion, the scaffold developed in the present work may prove to be more effective for the electrical stimulation of biological cells than metal-based stiff electrodes with limited biocompatibility[22]. Hence we believe that use of a small weight fraction of carbon nanotubes may justify its use in order to overcome the limitations of currently used metal-based electrodes for biological cell stimulation.

5. Conclusions

We have successfully manufactured electrically conductive biocompatible chitin/MWNT composite films using ionic liquid as a benign common solvent/dispersion medium. The non-covalent modification of MWNTs with carboxymethyl cellulose was shown to be effective in improving dispersion of MWNTs in ionic liquid. After achieving improved dispersion of MWNTs, chitin/MWNT composites with MWNT concentrations ranging from 0.01 to 0.1 mass fractions were manufactured. The 0.07 and 0.1 mass fraction chitin/MWNT composites were found to have high electrical conductivity and the 0.07 mass fraction chitin/MWNT composite scaffold was found to have the best combination of high electrical conductivity with good survival and proliferation of MSCs. Our results demonstrate the potential of electrically conductive, biocompatible, chitin/MWNT composites in regenerative medicine prepared using a benign solvent. These soft biocompatible and electrically conductive films may offer an attractive alternative to metal-based electrodes for the electrical stimulation of tissues.

Acknowledgements

Funding from ‘EPSRC Cross Disciplinary Feasibility Study’ and University of Bristol, Faculty of Engineering pump prime funding is gratefully acknowledged. The authors would like to acknowledge support from the Centre for Nanoscience and Quantum Information for the access of their material characterization facility. K. Koziol thanks the Royal Society and European Research Council for financial support. J. W Gilman acknowledges support by the Air Force Office of Scientific Research (AFOSR-MIPR, F1ATA02045G002).

Figure1

a: Schematic representation of the preparation of chitin/MWNT composite films.

b: The chitin and carbon nanotube composite films with different concentrations of MWNTs. The 0.07 and 0.1 weight fraction MWNT composites show visibly reduced optical transparency. (Scale bar is 130 μm for all membranes)

Figure 2a. Optical microscopy image of pristine MWNTs showing poor dispersion in EMI Ac. **2b.** Optical microscopy images of carboxymethyl cellulose modified MWNTs showing improved dispersion in EMI Ac

Figure 3: SEM of MWNTs dispersed in chitin films at low (Fig 3 a, c, e, and g) and high (Fig 3 b, d, f and h) magnification.

Figure 4: Wide-angle X-ray diffraction pattern of the lowest concentration (0.01) mass fraction and highest concentration (0.1) weight fraction of chitin/MWNT composite films.

Figure 5: FTIR spectra of the lowest concentration (0.01) weight fraction and highest concentration (0.1) weight fraction of chitin/MWNT composite films.

Figure 6: The electrical conductivity of chitin/MWNT composite films which shows excellent electrical conductivity for 0.07 and 0.1 weight fraction % of MWNT and chitin composites films.

Figure 7: Live and dead viability assay to test the viability of MSCs on the chitin/MWNT film scaffolds. Cells were seeded on the scaffolds and were stained with the Live/Dead viability stain. Green cells showed the number of live cells and red cells showed dead cells due to the excitation of florescent dye (calcein AM) at 490 nm. The images were obtained after 3 d (left column) and 14 d (right column) of cells seeding.

Figure 8: MTS assay quantifying MSC proliferation on different concentrations of chitin/MWNT films. The positive control in this case is the tissue culture plate (plastic). A cell count below 250 was disregarded from the analysis due to the inherent limitation of the assay. Data are given as mean + standard error.

Figure 1

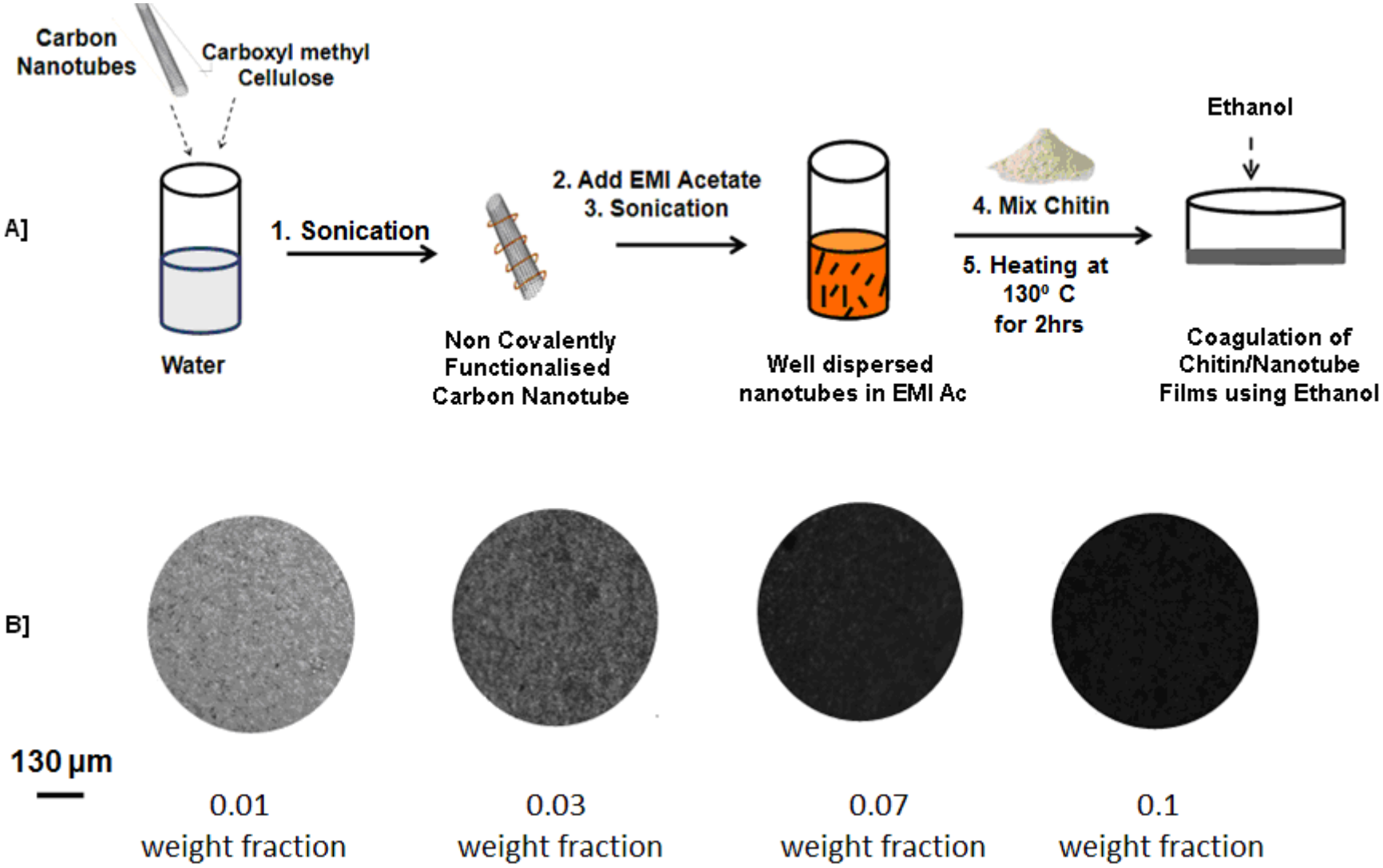
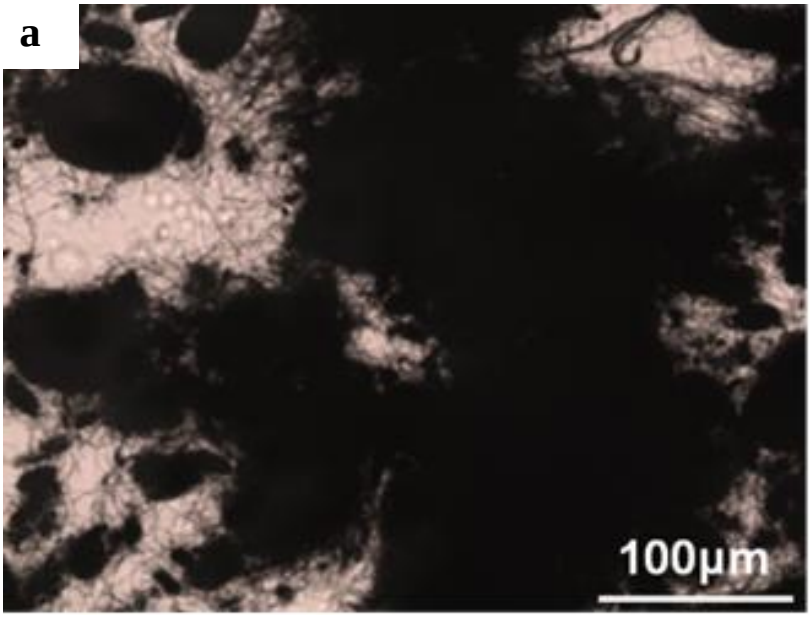


Figure 2



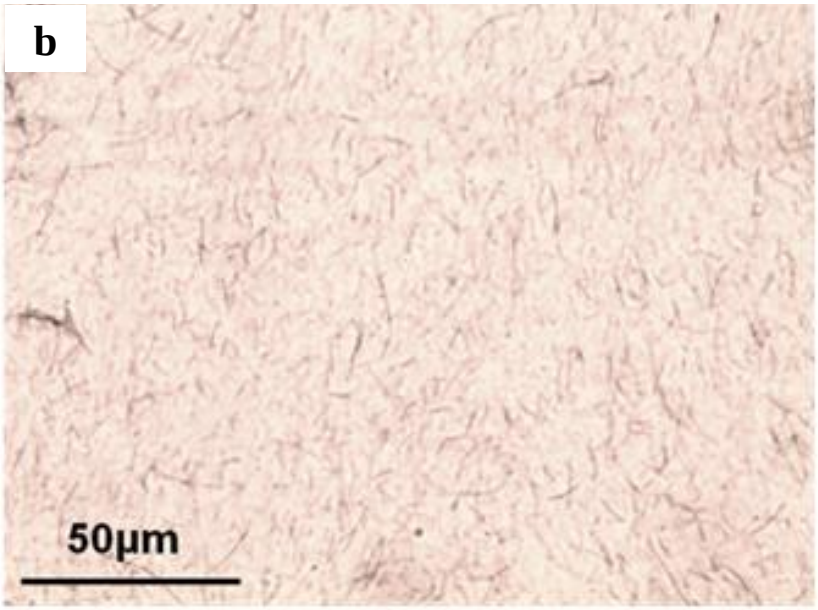


Figure 3

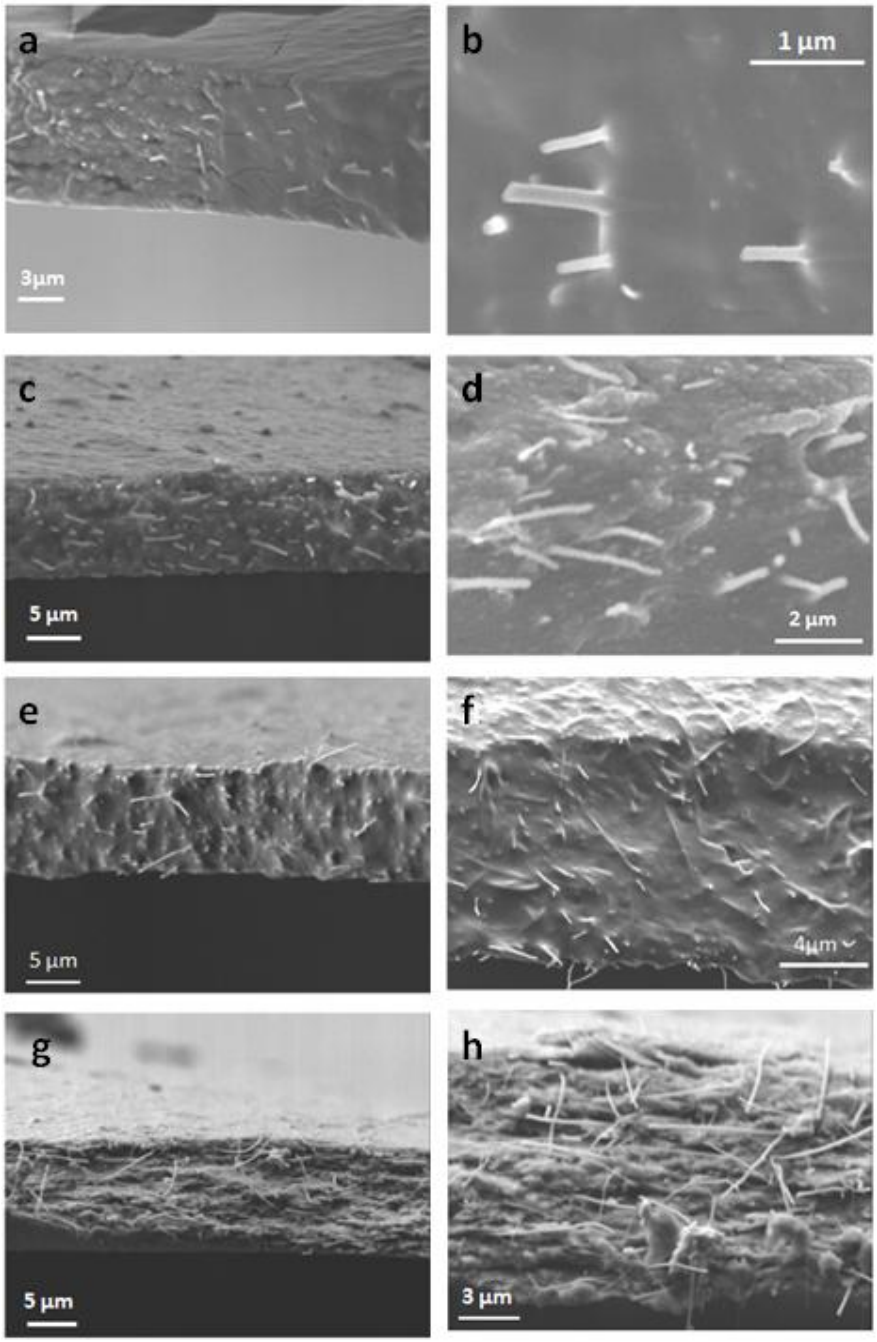
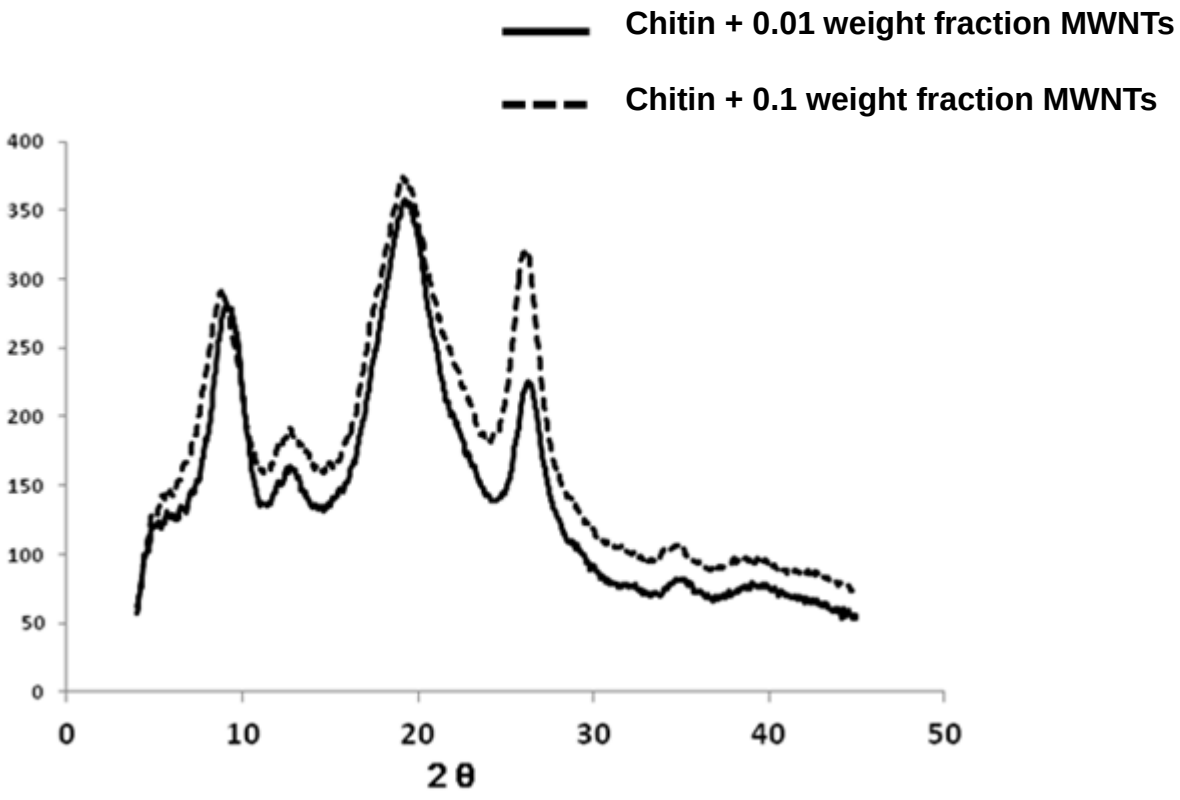


Figure 4



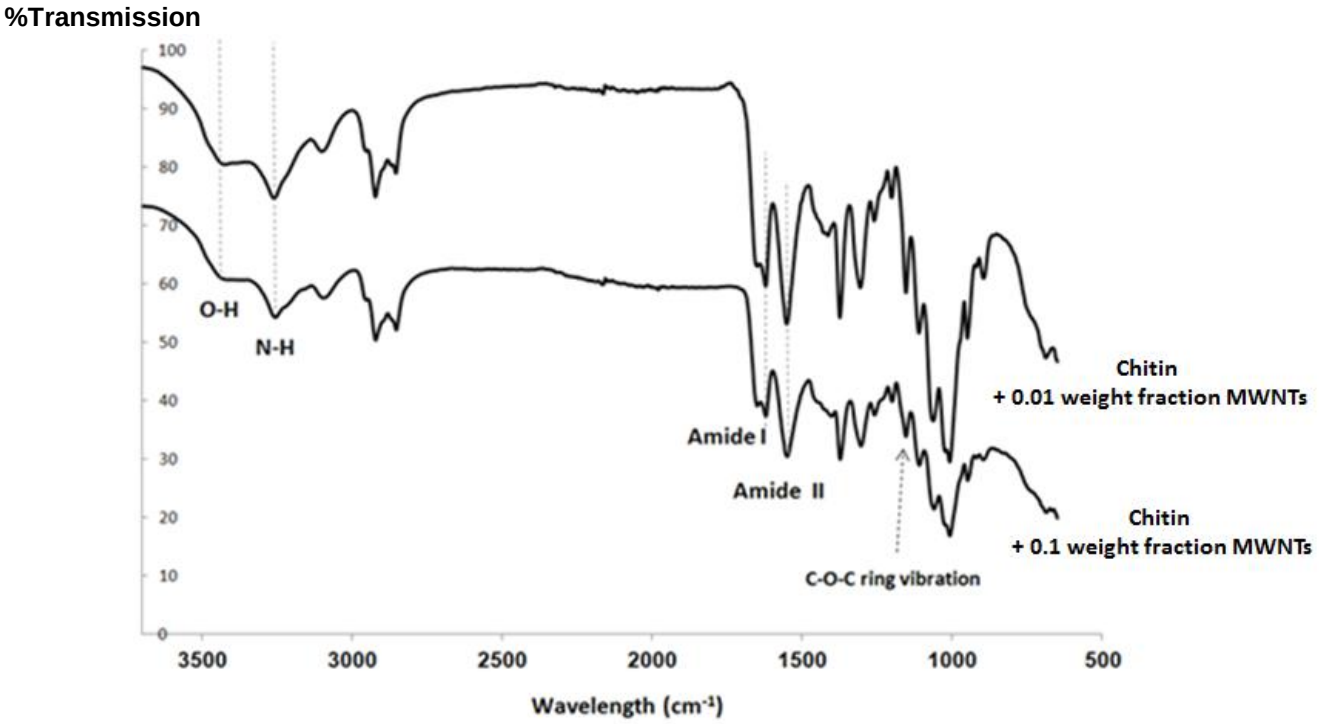


Figure 5

Figure 6

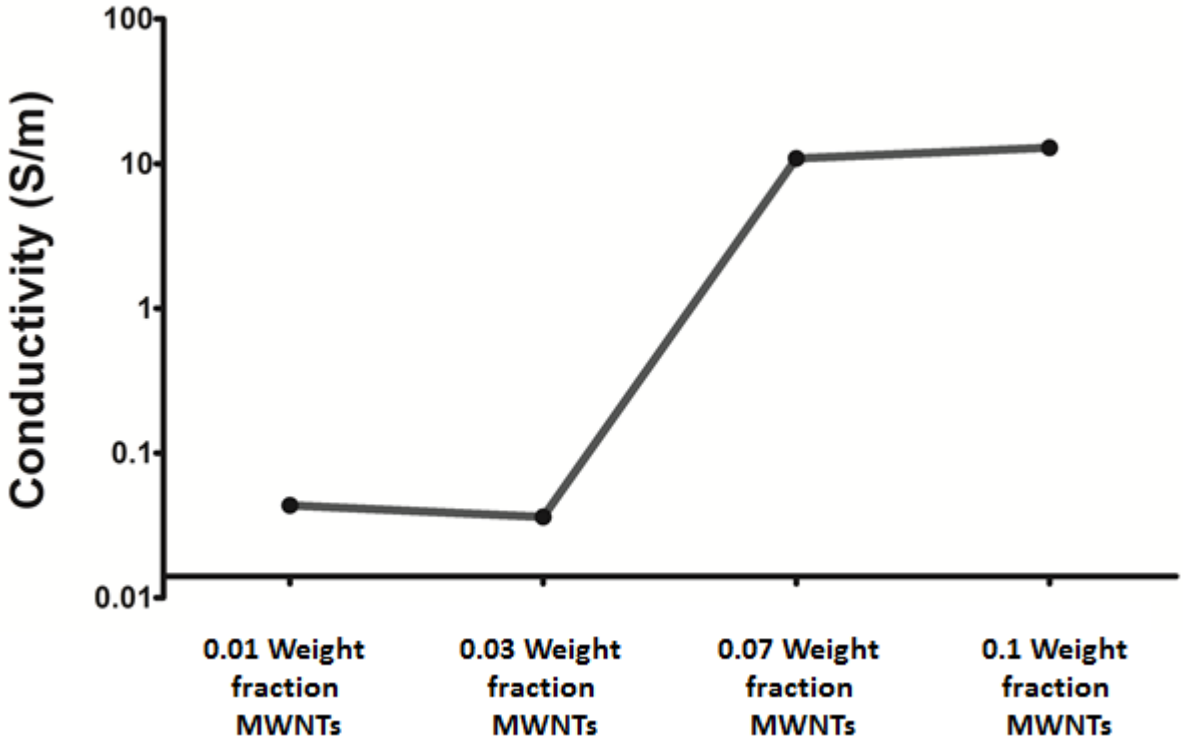


Figure7

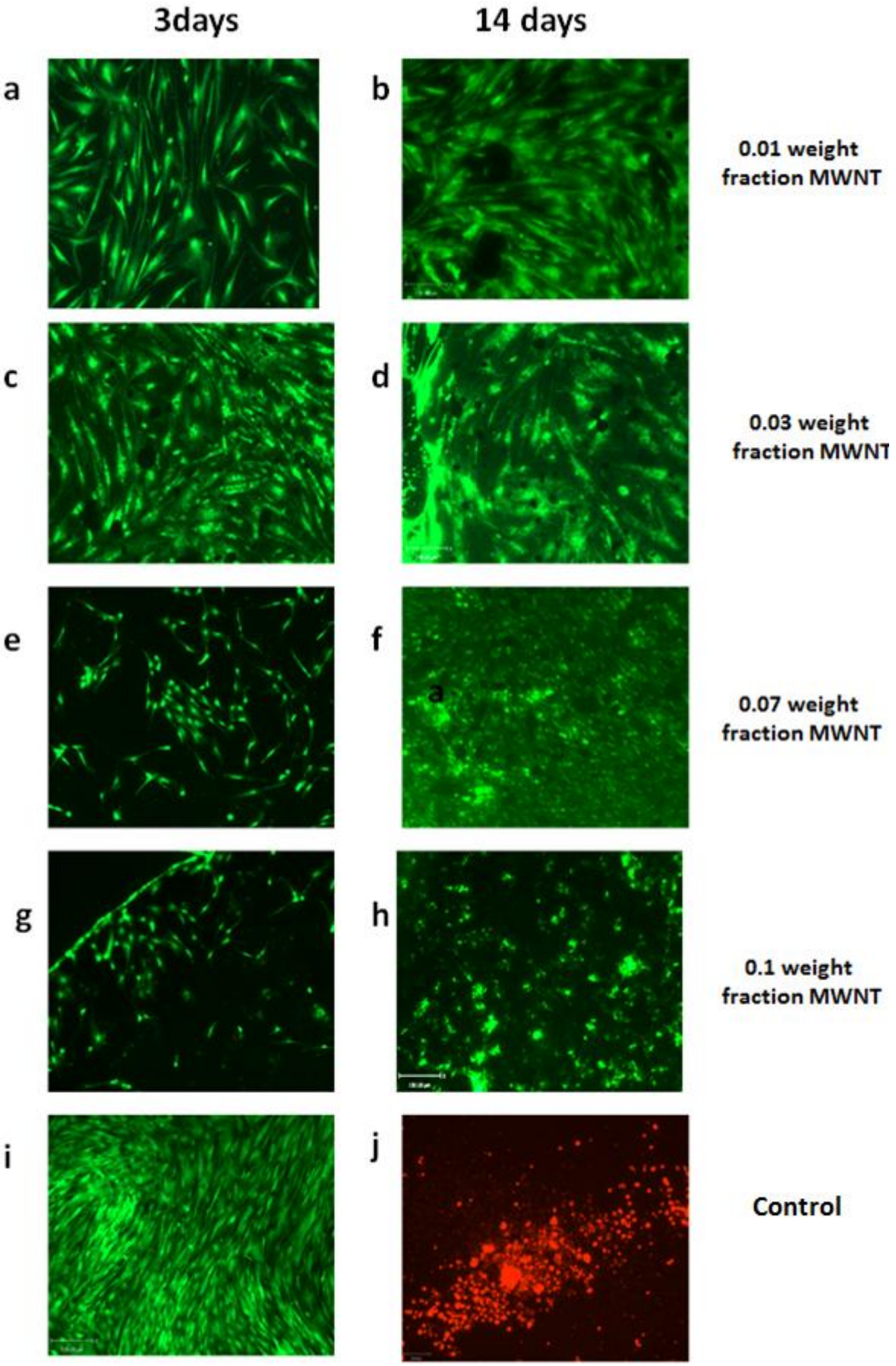
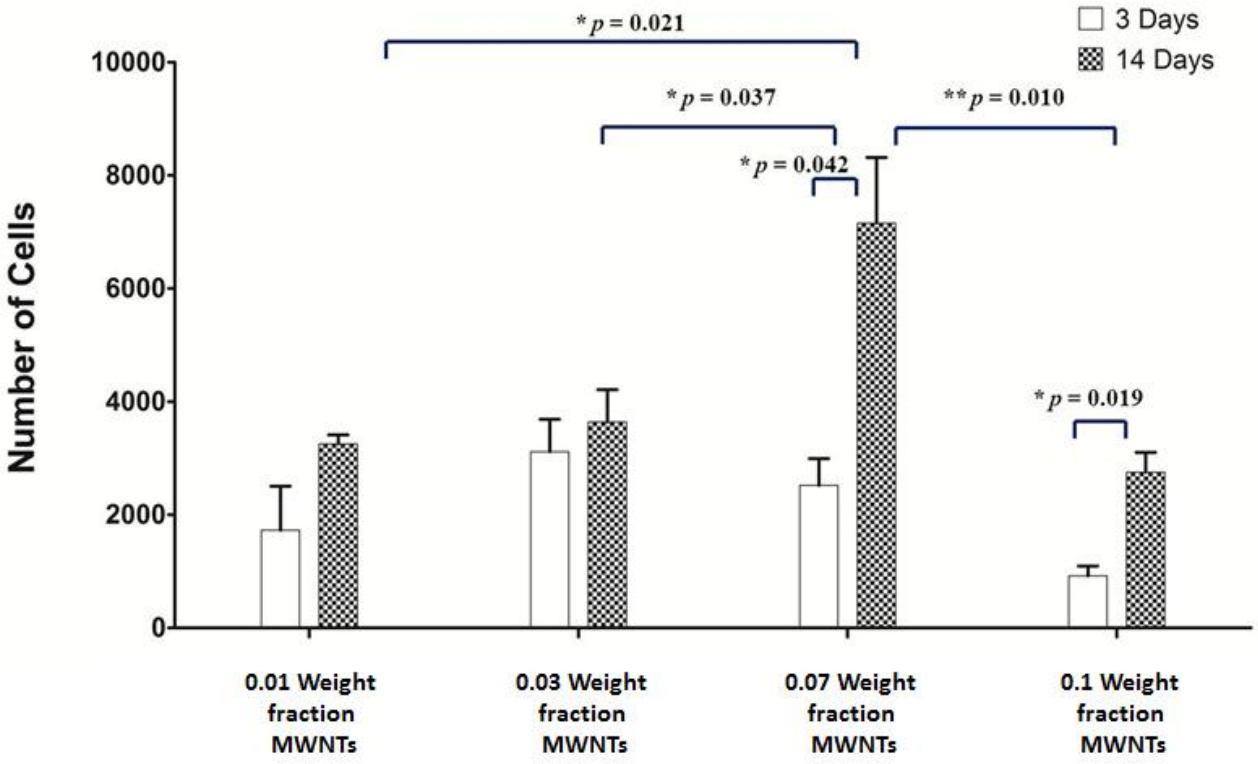


Figure 8



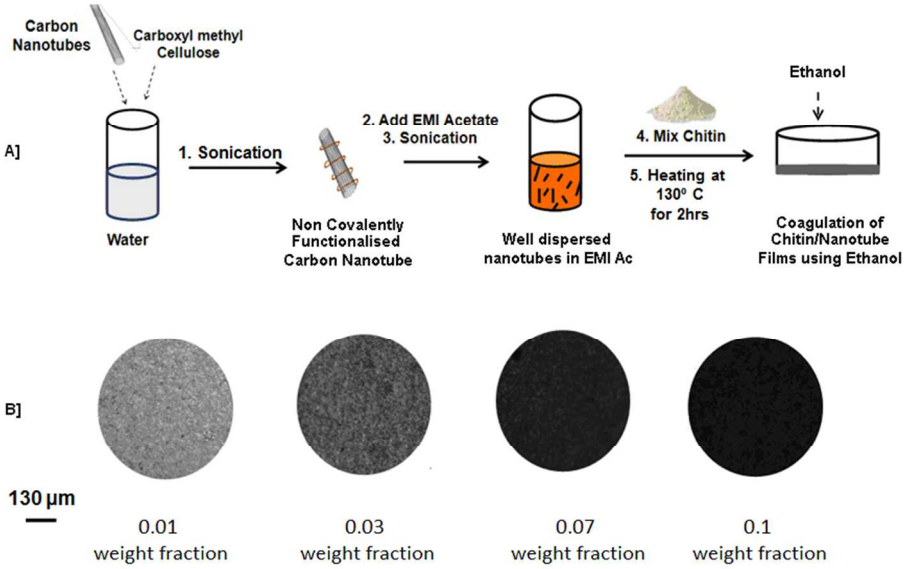
References

1. M. N. V. R. Kumar, *Reactive and functional polymer*, 2000, **46**, 1-27.
2. N. Nishi, A. Ebina, S. Nishimura, A. Tsutsumi, O. Hasegawa and S. Tokura, *Int J Biol Macromol*, 1986, **8**, 311-317.
3. L. P. Yan, J. M. Oliveira, A. L. Oliveira, S. G. Caridade, J. F. Mano and R. L. Reis, *Acta Biomater*, 2012, **8**, 289-301.
4. M. P. Prabhakaran, J. R. Venugopal and S. Ramakrishna, *Biomaterials*, 2009, **30**, 4996-5003.
5. D. M. Phillips, L. F. Drummy, D. G. Conrady, D. M. Fox, R. R. Naik, M. O. Stone, P. C. Trulove, H. C. De Long and R. A. Mantz, *Journal of the American chemical society*, 2004, **126**, 14350-14351.
6. H. B. Xie, S. B. Zhang and S. H. Li, *Green Chemistry*, 2006, **8**, 630-633.
7. S. D. Zhu, Y. X. Wu, Q. M. Chen, Z. N. Yu, C. W. Wang, S. W. Jin, Y. G. Ding and G. Wu, *Green Chemistry*, 2006, **8**, 325-327.
8. S. K. S. R. P.Swatloski, J. D. Holbrey,and R. D. Rogers, *Journal of the American chemical society*, 2002, **124** 4974 - 4975.
9. A. Takegawa, M. Murakami, Y. Kaneko and J. Kadokawa, *Carbohydrate Polymers*, 2010, **79**, 85-90.
10. Y. Qin, X. M. Lu, N. Sun and R. D. Rogers, *Green Chemistry*, 2010, **12**, 968-971.
11. Y. Wu, T. Sasaki, S. Irie and K. Sakurai, *Polymer*, 2008, **49**, 2321-2327.
12. S. S. Silva, A. R. C. Duarte, A. P. Carvalho, J. F. Mano and R. L. Reis, *Acta Biomaterialia*, 2011, **7**, 1166-1172.
13. B. B. Ding, J. Cai, J. C. Huang, L. N. Zhang, Y. Chen, X. W. Shi, Y. M. Du and S. Kuga, *Journal of Materials Chemistry*, 2012, **22**, 5801-5809.
14. E. Serena, E. Figallo, N. Tandon, C. Cannizzaro, S. Gerecht, N. Elvassore and G. Vunjak-Novakovic, *Exp Cell Res*, 2009, **315**, 3611-3619.
15. M. Yamada, K. Tanemura, S. Okada, A. Iwanami, M. Nakamura, H. Mizuno, M. Ozawa, R. Ohyama-Goto, N. Kitamura, M. Kawano, K. Tan-Takeuchi, C. Ohtsuka, A. Miyawaki, A. Takashima, M. Ogawa, Y. Toyama, H. Okano and T. Kondo, *Stem Cells*, 2007, **25**, 562-570.
16. E. Holt, P. K. Lunde, O. M. Sejersted and G. Christensen, *Basic Res Cardiol*, 1997, **92**, 289-298.
17. N. M. Geremia, T. Gordon, T. M. Brushart, A. A. Al-Majed and V. M. K. Verge, *Exp Neurol*, 2007, **205**, 347-359.
18. D. Khang, G. E. Park and T. J. Webster, *Journal of Biomedical Materials Research Part A*, 2008, **86A**, 253-260.
19. M. N. V. R. Kumar, *Reactive & Functional Polymers*, 2000, **46**, 1-27.
20. R. Jayakumar, T. Egawa, T. Furuike, S. V. Nair and H. Tamura, *Polym Eng Sci*, 2009, **49**, 844-849.
21. C. W. Lam, J. T. James, R. McCluskey and R. L. Hunter, *Toxicological Sciences*, 2004, **77**, 126-134.
22. N. A. Kotov, J. O. Winter, I. P. Clements, E. Jan, B. P. Timko, S. Campidelli, S. Pathak, A. Mazzatenta, C. M. Lieber, M. Prato, R. V. Bellamkonda, G. A. Silva, N. W. S. Kam, F. Patolsky and L. Ballerini, *Advanced Materials*, 2009, **21**, 3970-4004.
23. D. Tasis, N. Tagmatarchis, A. Bianco and M. Prato, *Chemical Reviews*, 2006, **106**, 1105-1136.
24. C. Singh, M. S. P. Shaffer, K. K. K. Koziol, I. A. Kinloch and A. H. Windle, *Chemical Physics Letters*, 2003, **372**, 860-865.
25. T. Takahashi, K. Tsunoda, H. Yajima and T. Ishii, *Japanese Journal of Applied Physics Part 1-Regular Papers Short Notes & Review Papers*, 2004, **43**, 3636-3639.
26. N. Minami, Y. J. Kim, K. Miyashita, S. Kazaoui and B. Nalini, *Applied Physics Letters*, 2006, **88**.
27. I. Riou, P. Bertoncini, H. Bizot, J. Y. Mevellec, A. Buleon and O. Chauvet, *Journal of Nanoscience and Nanotechnology*, 2009, **9**, 6176-6180.

28. R. Haggenmueller, S. S. Rahatekar, J. A. Fagan, J. H. Chun, M. L. Becker, R. R. Naik, T. Krauss, L. Carlson, J. F. Kadla, P. C. Trulove, D. F. Fox, H. C. DeLong, Z. C. Fang, S. O. Kelley and J. W. Gilman, *Langmuir*, 2008, **24**, 5070-5078.
29. C. Zhu, unpublished work.
30. W. Kafienah, S. Mistry, M. J. Perry, G. Politopoulou and A. P. Hollander, *Stem Cells*, 2007, **25**, 2460-2468.
31. L. A. Solchaga, K. Penick, J. D. Porter, V. M. Goldberg, A. I. Caplan and J. F. Welter, *Journal of Cellular Physiology*, 2005, **203**, 398-409.
32. S. Ifuku, M. Nogi, K. Abe, M. Yoshioka, M. Morimoto, H. Saimoto and H. Yano, *Biomacromolecules*, 2009, **10**, 1584-1588.
33. G. Cardenas, G. Cabrera, E. Taboada and S. P. Miranda, *Journal of Applied Polymer Science*, 2004, **93**, 1876-1885.
34. F. A. Al Sagheer, M. A. Al-Sughayer, S. Muslim and M. Z. Elsabee, *Carbohydrate Polymers*, 2009, **77**, 410-419.
35. R. Minke and J. Blackwell, *Journal of Molecular Biology*, 1978, **120**, 167-181.
36. B. Focher, A. Naggi, G. Torri, A. Cosani and M. Terbojevich, *Carbohydrate Polymers*, 1992, **17**, 97-102.
37. R. L. Lavall, O. B. G. Assis and S. P. Campana, *Bioresource Technology*, 2007, **98**, 2465-2472.
38. F. G. Pearson, R. H. Marchessault and C. Y. Liang, *Journal of Polymer Science*, 1960, **43**, 101-116.
39. M. Rinaudo, *Progress in Polymer Science*, 2006, **31**, 603-632.
40. R. Seoudi, A. M. A. Nada, S. Abd Elmongy and S. S. Hamed, *J Appl Polym Sci*, 2005, **98**, 936-943.
41. C. Zhong, A. Cooper, A. Kapetanovic, Z. H. Fang, M. Q. Zhang and M. Rolandi, *Soft Matter*, 2010, **6**, 5298-5301.
42. F. R. a. A. CA, *Hum Exp Toxicol* 2010. , **29**, 1038 – 1054.
43. T. P. Pham, C. W. Cho and Y. S. Yun, *Water Res*, 2010, **44**, 352-372.
44. M. J. Earle, J. M. S. S. Esperanca, M. A. Gilea, J. N. C. Lopes, L. P. N. Rebelo, J. W. Magee, K. R. Seddon and J. A. Widegren, *Nature*, 2006, **439**, 831-834.
45. J. Dupont and P. A. Suarez, *Phys Chem Chem Phys*, 2006, **8**, 2441-2452.
46. D. A. Fox, WH; Gilman, JW et.al, *Green Chemistry* 2003, **5**, 724-727.
47. Y. V. Nancharaiah and A. J. Francis, *Bioresour Technol*, 2011, **102**, 6573-6578.
48. H. Koga, T. Fujigaya, N. Nakashima and K. Nakazawa, *Journal of Materials Science-Materials in Medicine*, 2011, **22**, 2071-2078.
49. T. I. Chao, S. H. Xiang, C. S. Chen, W. C. Chin, A. J. Nelson, C. C. Wang and J. Lu, *Biochem Bioph Res Co*, 2009, **384**, 426-430.
50. S. D. McCullen, D. R. Stevens, W. A. Roberts, L. I. Clarke, S. H. Bernacki, R. E. Gorga and E. G. Loboa, *International Journal of Nanomedicine*, 2007, **2**, 253-263.
51. E. Jan and N. A. Kotov, *Nano Lett*, 2007, **7**, 1123-1128.
52. T. R. Nayak, L. Jian, L. C. Phua, H. K. Ho, Y. P. Ren and G. Pastorin, *Acs Nano*, 2010, **4**, 7717-7725.
53. S. Sirivisoot and B. S. Harrison, *Int J Nanomed*, 2011, **6**, 2483-2497.
54. S. I. Ertel, *Journal of Biomedical Materials Research*, 1994, **28**, 667-675.
55. B. I. Florea, C. Meaney, H. E. Junginger and G. Borchard, *Aaps Pharmsci*, 2002, **4**.
56. P. H. Mosier-Laclair S, Pomeroy G., *Foot Ankle Int.*, 2001, **22**, 247-251.
57. S. S. Rahatekar, M. Hamm, M. S. P. Shaffer and J. A. Elliott, *Journal of Chemical Physics*, 2005, **123**.
58. F. M. Du, R. C. Scogna, W. Zhou, S. Brand, J. E. Fischer and K. I. Winey, *Macromolecules*, 2004, **37**, 9048-9055.
59. J. K. W. Sandler, J. E. Kirk, I. A. Kinloch, M. S. P. Shaffer and A. H. Windle, *Polymer*, 2003, **44**, 5893-5899.
60. N. Lewinski, V. Colvin and R. Drezek, *Small*, 2008, **4**, 26-49.

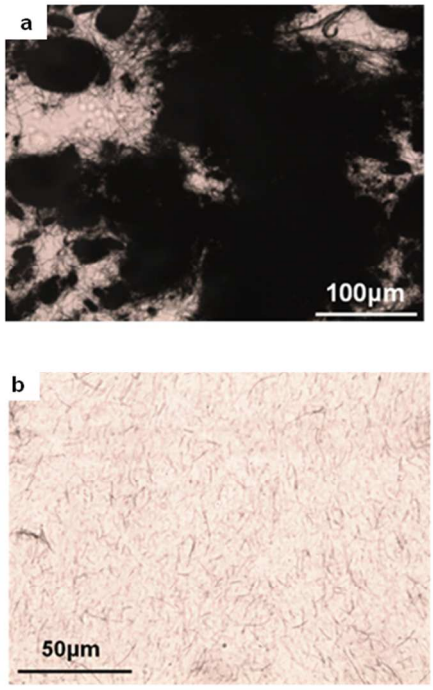
61. C. A. Poland, R. Duffin, I. Kinloch, A. Maynard, W. A. Wallace, A. Seaton, V. Stone, S. Brown, W. Macnee and K. Donaldson, *Nat Nanotechnol*, 2008, **3**, 423-428.
62. V. Lovat, D. Pantarotto, L. Lagostena, B. Cacciari, M. Grandolfo, M. Righi, G. Spalluto, M. Prato and L. Ballerini, *Nano Letters*, 2005, **5**, 1107-1110.
63. G. Cellot, E. Cilia, S. Cipollone, V. Rancic, A. Sucapane, S. Giordani, L. Gambazzi, H. Markram, M. Grandolfo, D. Scaini, F. Gelain, L. Casalis, M. Prato, M. Giugliano and L. Ballerini, *Nat Nanotechnol*, 2009, **4**, 126-133.

Figure 1



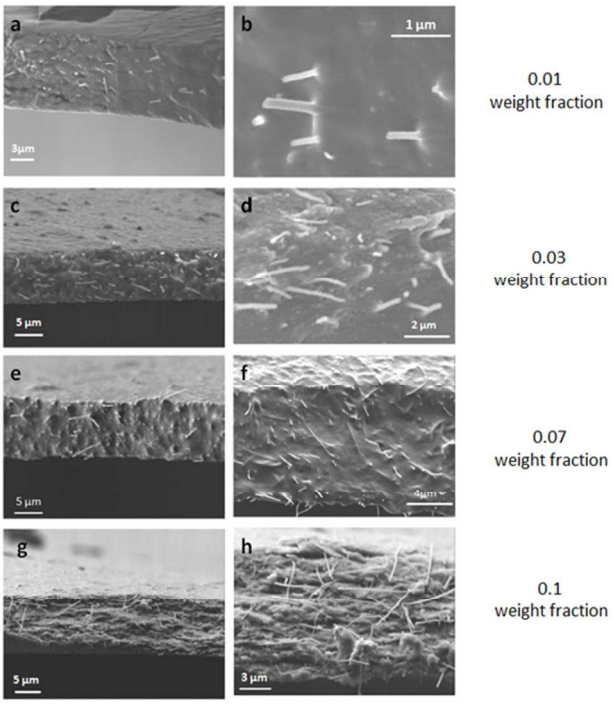
254x190mm (96 x 96 DPI)

Figure 2



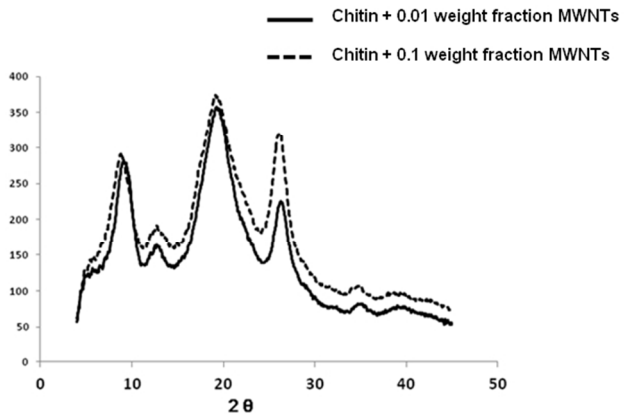
254x190mm (96 x 96 DPI)

Figure 3



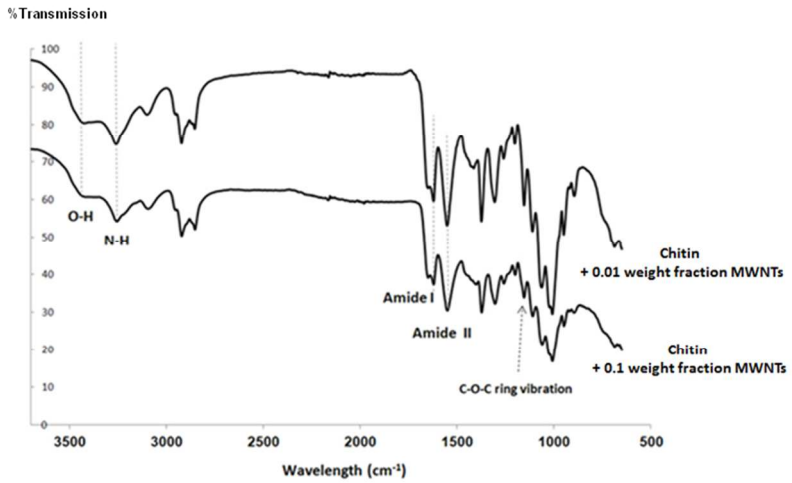
254x190mm (96 x 96 DPI)

Figure 4



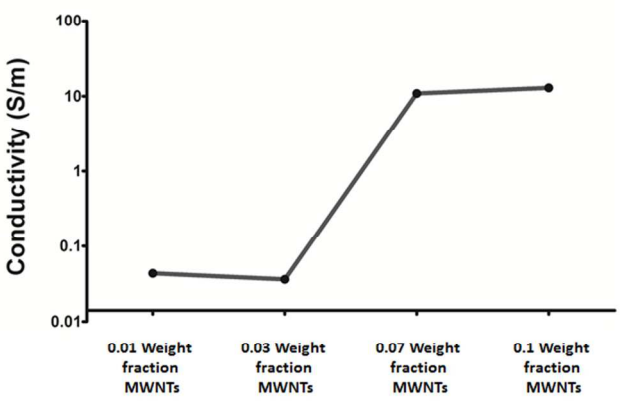
254x190mm (96 x 96 DPI)

Figure 5



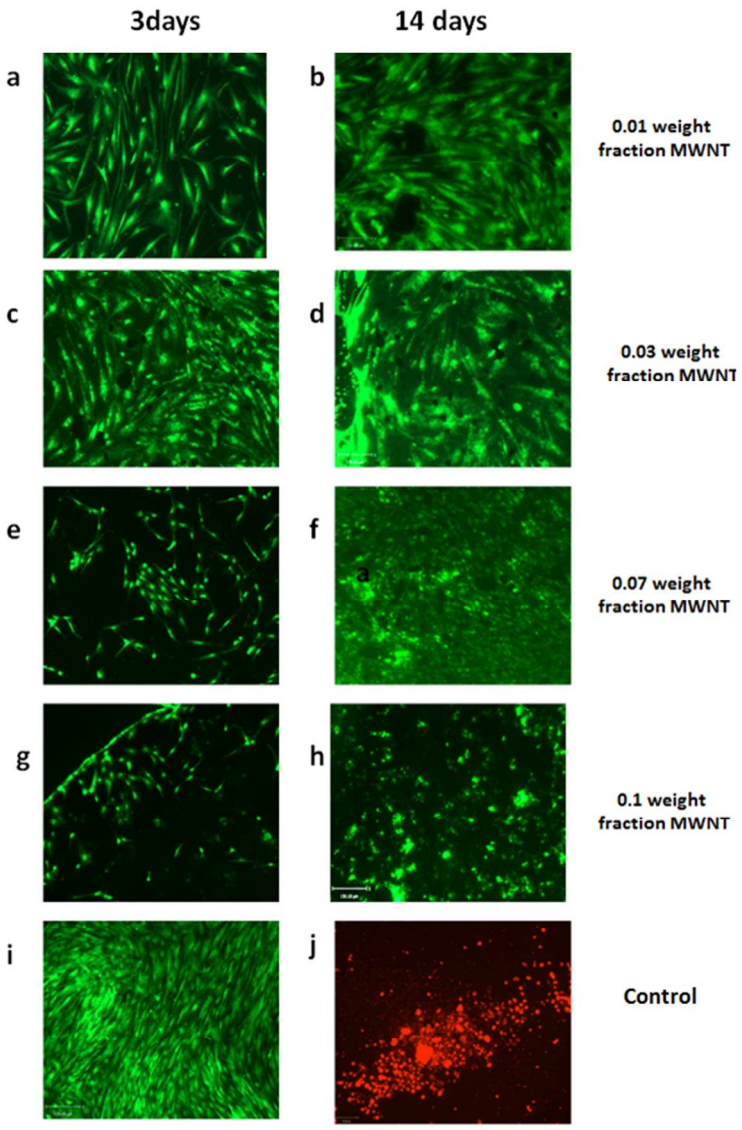
254x190mm (96 x 96 DPI)

Figure 6



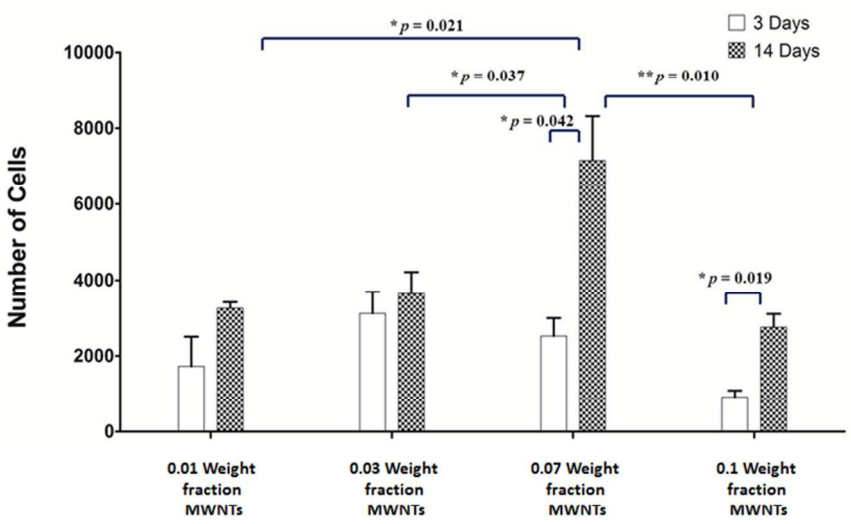
254x190mm (96 x 96 DPI)

Figure7



190x254mm (96 x 96 DPI)

Figure 8



254x190mm (96 x 96 DPI)