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Electrically Conductive MEH-PPV:PCL Electrospun Nanofibres for Electrical Stimulation of Rat PC12 Pheochromocytoma Cells

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Abstract. The purpose of this study was to prepare an electrically conducting poly[2-methoxy-5-(2-ethyl-hexyloxy)-1,4-phenylene vinylene] (MEH-PPV) based nanofibrous scaffold and to investigate the synergetic effect of nanofibre structure and electrical stimulation on neuronal growth for possible use in nerve repair. Nanofibres were produced by electrospinning of blended MEH-PPV with polycaprolactone (PCL) at a ratio of 20:80, 40:60, 50:50 and 60:40 (v/v). A better electrical conductivity was achieved by using core-sheath structured nanofibres of PCL (core) and MEH-PPV (sheath) produced using the coaxial electrospinning technique. The highest electrical conductivity was observed in the core-sheath nanofibres, while it

increased with increasing concentration of MEH-PPV for the blended electrospun nanofibres. The biocompatibility of the electrospun nanofibres was confirmed by MTS and live-dead staining assays using 3T3 fibroblasts and a neuronal rat pheochromocytoma (PC12) cell line. Beta (III) tubulin immunocytochemistry showed that PC12 cells differentiated into sympathetic neurons on these porous and stiffer electrospun nanofibres coated with collagen I. Improved cell morphology and attachment on the collagen I coated electrospun meshes has been confirmed by SEM analysis. Significant enhancement in neurite formation and neurite outgrowth of PC12 cells on the conductive scaffolds under electrical potential of 500 mV/cm for 2 h/day suggest the potential use of these scaffolds for nerve repair.

Keywords: Nerve repair, Electrospinning, Electrical stimulation (ES), MEH-PPV, PC12

1. Introduction

Neural regeneration following injury remains a significant challenge with major implications for patient quality of life. Complications associated with damage to sensory and motor neural pathways may result in severe pain and malfunction of organs including the digestive tract, heart and sex organs.¹ Current clinical treatment for peripheral nerve injury are surgical end to end anastomoses and autologous nerve grafts. There is no effective treatment for damage to the central nervous system (CNS) or for absolute nerve tissue regeneration.² There are several disadvantages to autografting and allografting, including loss of function at the donor site, mismatch of nerve cable dimensions between the donor graft and recipient nerve, and the need for multiple surgeries.²⁻⁴ Thus, at present there is no universally accepted treatment available for nerve regeneration and in the majority of cases it is still not possible.

Neural tissue engineering as a combination of cell, scaffold, and bio/chemical/physical cues could be an alternative for the above strategies. Work in this area has led to the development of engineered nerve guidance channels (NGCs) using collagen and synthetic polymers with a variety of well-defined features such as porosity, biocompatibility, biodegradability, and resistance to infections.^{5,6} However, none of the above strategies have

produced entirely satisfactory results. A successful neural tissue engineering approach should also accelerate the rate of nerve regeneration. Patients undergoing immediate peripheral nerve repair (PNR) are subject to a lengthy denervation period of the distal target, given that the rate of regeneration approaches 1 mm/day in humans. Consequently, this can result in considerable atrophy of the denervated tissue by the time regenerating axons have arrived at the tissue. So, accelerating the rate of nerve regeneration can result in a better functional outcome for the denervated tissue.

Electrical stimulation (ES), in that respect, is one of the potential methods to improve the rate of nerve regeneration and the restoration of function.⁷⁻¹⁰ The concept of using ES for nerve regeneration is based on the fact that the bioelectricity plays an integral role in maintaining normal biological functions via cranial, spinal and peripheral neural networks which signal for example muscle contraction and wound healing in bone, cartilage, skin, connective tissue.^{2,13,14} ES activates neurons through membrane depolarization which ultimately promotes the speed and accuracy of motor and sensory axon regeneration both *in vitro* and *in vivo*. Specifically, the electrical signal in the peripheral nervous system accelerates axonal regeneration and elongation and enhances expression of neurotrophic factors and the biological activity of Schwann cells.^{1,9}

In this context, conducting polymers (CPs) can be used to build scaffolds that offer excellent control over the level and duration of the electrical stimulus preferentially localized to the target area.^{2,7} Intended for nerve regeneration, CPs have a higher charge injection limit with improved charge-discharge characteristics leading to enhanced charge transportation to cells for membrane depolarisation. This, in turn, can improve the adhesion and proliferation of nerve cells including the promotion of axonal growth.^{15,16} Additionally, CPs possess very good electrical and optical properties, a high conductivity/weight ratio and can be made biocompatible, biodegradable and porous.^{9,17,18} The chemical, electrical and physical properties of CPs can be altered to suit specific applications by incorporating different

functionality even after synthesis. Considering these versatility, CPs such as polypyrrole (PPy), polythiophene (PT), polyaniline (PANI) and poly(3,4-ethylenedioxythiophene) (PEDOT)) have been investigated since the discovery of CP in 1977 for numerous applications such as microelectronics,^{19,20} polymer batteries,^{19,21} supercapacitors,²⁰ actuators,^{18,19,22} and in biomedical applications as microsurgical tools,^{18,23} biosensors,^{18,19} drug delivery systems,^{9,18} and in tissue engineering.^{24,25} These CPs were studied with various cell types including endothelial cells,²⁶ rat pheochromocytoma (PC12) cells,²⁷ cardiac myoblasts,²⁴ neurons and support cells (i.e., glia, fibroblasts) associated with dorsal root ganglia (DRG),^{27,28} primary neurons,^{29,30} keratinocytes,³¹ and mesenchymal stem cells (MSC).³² The beneficial effect of electrical stimulation (ES) on neurite formation and neurite outgrowth has been shown using polypyrrole (PPy), polythiophene (PT), polyaniline (PANI) and poly(3,4-ethylenedioxythiophene) (PEDOT) with PC12 cells,^{13,27} retinal ganglion cell (RGC),¹⁵ dorsal root ganglion (DRG),²⁷ and nerve stem cells.¹³ However, ES of cells using polymers other than PPy is limited. One of the major problems associated with these polymers is poor solubility.³³ One potential method by which to produce a neural guidance mesh is using an electrospinning fabrication technique, which can yield non-woven, three dimensional, porous and nanofibrous scaffolds with suitable mechanical properties to mimic the native extracellular matrix (ECM).^{6,11,12}

In this study, we explored a new CP, MEH-PPV for use as a conductive nanofibrous biomaterial scaffold. MEH-PPV has not been assessed for tissue engineering applications before, although it offers an interesting property for biological application since it allows the immobilization of biomolecules due to its high density hole-traps.³⁴ MEH-PPV is a p-type semiconducting polymer that has low conductivity due to its low hole and electron mobilities,³⁵ and is currently used in electronic applications such as LEDs,³⁶ and photovoltaic cells.³⁷ However, suitable doping can improve its conductivity for desired applications. For example, Shin Sakiyama *et al.* demonstrated remarkable improvement in the conductivity of

MEH-PPV using FeCl_3 (p-type dopant) and Cs_2CO_3 (n-type dopant).³⁸ Despite its better solubility in common organic solvent when compared to the other CPs discussed above, direct electrospinning of MEH-PPV to a uniformly distributed, one-dimensional nanofibrous mat free from bead formation is difficult.³⁹ Blending with other natural or synthetic polymers that are biocompatible, biodegradable and easily electrospinnable may be a means to overcome this limitation. The aliphatic linear polyester, polycaprolactone (PCL) is a potential candidate, with attractive electrospinnability due to its good rheological and viscoelastic properties.⁴⁰ Electrospun PCL scaffolds have already been widely studied for various tissue engineering applications owing to their biocompatibility, biodegradability and mechanical properties.⁴¹

In the present study, we report for the first time the electrospinning of a blend of FeCl_3 doped MEH-PPV with PCL and an investigation of the impact of various composition ratios on the physical, chemical and biological properties of the resulting electrospun fibres. Here, we also report the coaxial electrospinning of conductive core-sheath nanofibres of PCL (core) and MEH-PPV (sheath) at different flow rates to obtain a more conductive scaffold for effective electrical stimulation of cells. The flowchart of the present research is shown with the help of a schematic illustration in Figure 1. The aim of this study is to investigate the combined effect of nanofibre structure and electrical stimulation through these electrospun conductive nanofibre meshes on neurite formation and neurite outgrowth using PC12 cells. If successful MEH-PPV based scaffolds could ultimately be used to design nerve guidance channels and bridge the gap between two damaged nerves.

2. Materials and Methods

2.1 Materials

MEH-PPV (M_w 150,000-250,000), PCL (M_w 80,000), Chloroform ($\geq 99.5\%$) Dimethylformamide (DMF, $\geq 99.8\%$), Dichloromethane (DCM, $\geq 99.8\%$), Iron(III) chloride (FeCl_3 , anhydrous, powder, $\geq 99.99\%$) were purchased from Sigma-Aldrich, UK and were

used as received without further purification. Dulbecco's Modified Eagle's Medium (DMEM, Gibco, UK), fetal bovine serum (FBS, Gibco, UK), trypsin-EDTA solution (Sigma-Aldrich, UK) and penicillin-streptomycin (10,000 U/mL, Gibco, UK) were purchased from Sigma-Aldrich for NIH 3T3 cell culture. RPMI-1640 media (Sigma-Aldrich, UK), horse serum (Hyclone), nerve growth factor (NGF- β from rat, Sigma-Aldrich, UK) and Collagen I (Type I solution from rat tail, BioReagent, Sigma-Aldrich, UK) were procured for PC12 cell culture. CellTiter 96® Aqueous One Solution Cell Proliferation Assay (MTS: [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium salt]) was purchased from Promega UK for the MTS assay. Primary antibody Rb pAb to anti beta III tubulin (ab18207, Abcam, UK), secondary antibody Goat pAb to Rb IgG Alexa fluor 488 (ab150077, Abcam, UK), 4',6-Diamidino-2-Phenylindole, Dihydrochloride (DAPI, Molecular Probes, UK), bovine serum albumin (BSA, Sigma-Aldrich, UK), normal goat serum (Sigma-Aldrich, UK), glycine (Acros Organics, UK), tween PBS (Pierce™ 20X PBS Tween™ 20 Buffer, Thermo Fisher Scientific, UK) were purchased for immunocytochemistry.

2.2 Analytical techniques

Morphological characterisation of the electrospun nanofibres and cultured PC12 was carried out using a Zeiss SIGMA FEG-SEM Scanning Electron Microscope [FEG-SEM]. Transmission electron microscopy was carried out using a TECNAI G2 20 S-TWIN (200kV); Resolution: 2.4Å, FEI COMPANY, USA, transmission electron microscope (TEM). XPS was performed using an ESCALAB 250 Xi system (Thermo Scientific) equipped with a monochromated Al K α X-ray source. Uniform charge neutralization was provided by beams of low-energy (≤ 10 eV) Ar⁺ ions and low-energy electrons guided by the magnetic lens. The standard analysis spot of ca. 900×600 μm^2 was defined by the microfocused X-ray source. Full survey scans (step size 1 eV, pass energy 150 eV, dwell time 50mS and 3 scans) and narrow scans (step size 0.1 eV, pass energy 20 eV, dwell time 100 mS and 5 scans) of the C1s

(BE ~285 eV) regions were acquired from three separate areas on each sample. Data were transmission function corrected and analysed using Thermo Advantage Software (Version 5.952) using a smart background. FTIR spectra were recorded using a Nicolet Impact I-410 Spectrometer (SpectraLab Scientific Inc., USA). Confocal microscopy was carried out using Leica TCS SP5 Confocal Laser Scanning Microscope [CLSM] (Leica Microsystems, UK). For stability test, SEM was performed using JSM 6390LV, JEOL, Japan.

2.3 Doping of MEH-PPV

MEH-PPV was doped using FeCl_3 as a p-type dopant as reported by Shin Sakiyama et al.³⁸ Briefly, FeCl_3 was dissolved in dehydrate ethanol at a concentration of 3 mg/mL and kept stirring overnight at 50°C overnight in a laminar hood. 0.5 wt% MEH-PPV was dissolved in a solution mixture of chloroform and DMF of 60:40 (v/v). The dopant solution was added to MEH-PPV solution at a concentration of 2 wt% against the polymer and stirred for 30 min. The resultant solution of MEH-PPV containing dopant FeCl_3 was used in electrospinning.

2.4 Electrospinning of MEH-PPV:PCL blend

PCL was dissolved in a solution of DCM and DMF (60:40 v/v) at a concentration of 14 wt%. FeCl_3 doped MEH-PPV solution was mixed with PCL solution at four volume ratios of 20:80 (v/v), 40:60 (v/v), 50:50 (v/v) and 60:40 (v/v). MEH-PPV and PCL solutions were stirred for 1-2 h at 100-150°C to obtain a complete dispersed solution for electrospinning. For electrospinning, the mixed polymer solution was fed into a 10 mL standard plastic syringe equipped with a 30-gauge stainless steel needle and electrospun using a syringe pump (KDS 200, KD Scientific Inc., USA) at a potential of 18 kV from a high voltage power supply (Spellman, UK) and flow rate of 0.5 mL/h. The schematic of the electrospinning set up was shown in Figure S1(a). Electrospun fibres were collected on an aluminium foil wrapped copper plate at a distance of 16 cm from the syringe needle tip. The as-spun nanofibrous meshes were air dried overnight to remove the residual solvent and carefully removed from

the aluminium foil for further investigation. All experiments were conducted at 18-22°C with an average humidity of 60-65%.

2.5 Coaxial electrospinning of MEH-PPV:PCL

For coaxial electrospinning, both PCL (core) solution and MEH-PPV (sheath) solution, prepared as described above, were fed into two 5 mL standard plastic syringes separately attached to a coaxial spinneret. The schematic of coaxial electrospinning is shown in Figure S1 (b). The flow rate was adjusted using a dual syringe infusion pump. The coaxial spinneret was connected to the same electrical potential, provided by a high voltage power supply. The coaxial electrospinning was carried out at two flow rates of 0.6 mL/h and 1 mL/h at an applied voltage of 20 kV using a 15 cm needle tip to collector (copper plate wrapped with aluminium foil) distance. After electrospinning, the deposited meshes were air dried for overnight and carefully removed from the aluminium foil. All experiments were conducted at a temperature of 18-22°C with an average humidity of 60-65%.

2.6 Stability test

To investigate the degradation and stability, all the electrospun meshes were incubated in a physiological solution of phosphate buffered saline (PBS, pH =7.4) at 37°C for 45 days as reported elsewhere.²⁷ Degradation and stability of the electrospun meshes were evaluated by SEM and measurements of current-voltage (I-V) characteristics after 45 days of incubation. For SEM images, the samples were washed with deionized water twice to remove salts and air dried. Measurements for I-V characteristics were performed three times for each sample.

2.7 Sample preparation for cell culture

All electrospun meshes were cut using a biopsy punch in a circular shape with diameters of 5 mm for cytotoxicity testing and 10 mm for immunostaining and cell morphology study. For electrical stimulation, electrospun meshes of diameter 15 mm were used. Since PC12 cells

adhere poorly to tissue culture plastic, 96 well tissue culture plate was coated with 0.01% collagen I in 0.1 M acetic acid for cytotoxicity tests. Electrospun meshes with and without collagen I coating were used for preliminary immunochemistry, whereas the electrical stimulation experiment was performed with collagen coated electrospun meshes only. Before all cell culture experiments, all electrospun meshes were kept in sterile PBS for 24 h and sterilised under UV light for 1h each side of the mesh.

2.8 Cell culture

A PC-12 (ATCC® CRL-1721™) cell line (P3) was cultured in RPMI-1640 media (Sigma-Aldrich) supplemented with 10% horse serum (Hyclone), 5% FBS (Hyclone) and 1% penicillin-streptomycin solution (Sigma-Aldrich). Cells were incubated at 37°C in 5% CO₂. Cells were passaged weekly using a 0.25% trypsin–EDTA solution (Sigma). The growth medium was changed to differentiating medium containing RPMI-1640 supplemented with 1% horse serum, 1% penicillin-streptomycin solution and 100 ng/mL nerve growth factor (NGF, Sigma-Aldrich) for neuronal differentiation after 24 h of culture.

A mouse embryonic 3T3 fibroblast cell line (P15), NIH 3T3 (ATCC® CRL-1658™) was used to investigate the impact of the electrospun meshes on cell viability. Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS and 1% penicillin-streptomycin (Pen Strep) and were incubated at 37°C in 5% CO₂. Cells were passaged weekly using a 0.25% trypsin–EDTA solution (Sigma).

2.9 MTS proliferation assay

The MTS proliferation assay was carried out to evaluate the cytotoxicity of the electrospun meshes following incubation with 3T3 fibroblasts and PC12 cells. Metabolically active cells are capable of reducing a tetrazolium compound into a water soluble formazan product. Non-viable cells rapidly lose their ability to reduce MTS. Therefore, the production of the coloured formazan product is proportional to the number of viable cells.⁴² 3T3 cells were seeded on

different substrates at a concentration of 5×10^3 cells/well in a 96 well plate for the MTS assay. PC12 cells were cultured in growth medium at a concentration of 1×10^4 cells/well in direct contact with the electrospun meshes. Cell viability after incubation of 24, 48 and 72 h on the materials was quantified using CellTiter 96® AQueous One Solution containing 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt) (MTS). The cultured materials were washed with sterile PBS twice, and MTS reagent diluted 1:5 in media was added directly to all the wells except the blank. Plates were incubated for 2 h at 37°C and optical density (OD) was measured at 490 nm. All experiments were repeated 4 times.

2.10 Beta tubulin immunocytochemistry

Immunocytochemistry was performed using neuronal marker beta III-tubulin to confirm the differentiation of the PC12 cells.⁴³ PC12 cells were cultured on uncoated and collagen I coated electrospun meshes in differentiating medium at a concentration of 1×10^5 cells/well for 7 days. Cells were washed in PBS, fixed in 100% methanol for 5 mins, washed thrice in PBS then incubated with blocking and permeabilizing solution of 1% bovine serum albumin (BSA, Sigma-Aldrich), 10% (v/v) normal goat serum (Sigma-Aldrich) and 0.3 M glycine (Acros Organics) in 0.1% (v/v) tween PBS for 1 h. The cells were incubated with primary antibody Rb pAb to anti beta III tubulin (ab18207, Abcam, UK), at a concentration of 5 µg/mL overnight at 40°C, washed thrice in PBS then incubated with secondary antibody Goat pAb to Rb IgG Alexa fluor 488 (ab150077, Abcam, UK) at a concentration of 2 µg/mL for 1h at room temperature. The cells were stained with DAPI for nuclei counterstaining and imaged using confocal microscopy.

2.11 Cell adhesion test

Cell proliferation and differentiated PC12 morphology on uncoated and collagen coated electrospun meshes were evaluated using SEM after 7 days of culture. All electrospun mesh

seeded with PC12 cells were rinsed with phosphate buffer saline (PBS) and fixed with 4% glutaraldehyde solution for 45 min at 4°C. Cell seeded scaffolds were then dehydrated by incubation with serially increasing concentrations of ethanol beginning with incubation in 30% ethanol for 1 h then, 50%, 60%, 70%, 80%, 90% and absolute ethanol for 10 min each. The dehydrated cell seeded scaffolds were air dried overnight prior to SEM analysis.

2.12 Electrical stimulation of PC12 cells

Electrical stimulation of PC12 cells was performed on collagen coated electrospun meshes according to the experimental condition as previously reported but with a slight modification.^{27,44,45} The electrical stimulation experiment was set up as shown in the schematic in Figure S2 (a). Briefly, an Ag wire was connected to a 15 mm diameter collagen coated electrospun mesh and was used as the working electrode (WE). The electrospun meshes connected to the Ag wires were fixed in a 24 well tissue culture plate. Thincert cell culture inserts (24 Well ThinCert™ Cell Culture Inserts, Greiner, New Zealand) with an inner diameter of 8.4 mm, height of 16.25 mm and working volume 0.4 mL - 1.2 mL, were fixed onto the electrospun meshes in a 24 well plate after removing the bottom membrane. The edges of the insert were sealed using poly(dimethylsiloxane) (PDMS, SYLGARD®184, Sigma) to prevent any direct contact between the cell culture medium and the Ag wire connected to the electrospun meshes. A Pt wire placed in the cell culture medium, at a distance of 1 cm from the WE, was used as counter electrode (CE), as shown in Figure S2 (a). The electrospun meshes were washed thrice with sterile deionized water and incubated in sterile PBS solution overnight. The whole assembly was sterilized under UV for 3 h. PC12 cells were cultured at 1×10^3 cells/well in growth medium, and after 24 h of culture, growth medium was replaced with differentiating medium containing NGF. Then, a constant electrical potential of 500 mV/cm was applied across the electrodes for 2 h/day for 3 consecutive days using a portable bipotentiostat (EmStat Blue, PalmSens BV, Netherlands).

The electrical stimulation was carried out by a double pulsed potential chronoamperometric technique in an incubator [Figure S2 (c)]. Current signal during electrical stimulation of PC12 cells on a random electrospun mesh was shown in Figure S7. The electrically stimulated PC12 cells were cultured for another 72 h without electrical stimulation. For comparison, PC12 cells on all the electrospun meshes without electrical stimulation were also cultured under the same condition for 7 days treated as control. The differentiating medium was changed every 2 days during the experimental period. Furthermore, the electrical stimulation was also applied to the PC12 cells cultured on the electrically conductive materials in the absence of NGF for the same duration to check the effect of electrical potential on neural differentiation of PC12 cells.⁴⁶ All the experiments were performed thrice.

2.13 Image analysis

The number of PC12 cells on the sample meshes was determined by counting nuclei stained with DAPI dye from confocal images. The total number of cells were counted in 3 fields of view (top, centre and down) for each of three repeat samples per substrate type. Neurite length was measured as a linear distance between the cell junction and the tip of a neurite. For PC12 cells, data was collected for neurite lengths at least as long as twice the diameter of the cell body.^{27,47} Neurite outgrowth was reported in terms of neurite length per cell (for cells that expressed at least one neurite) and median neurite length. Also, the percentages of PC12 cells with neurites and the numbers of neurites per cell (for cells that expressed at least one neurite) were calculated. In the case of neurites with an ambiguous origin, the longest neurite was retained for the measurements to prevent repeated sampling of the same neurite segment within each image.

2.14 Statistical analysis

All experiments for statistical analysis were repeated with a minimum of $n = 3$. Statistical analysis was performed using one-way analysis of variance (ANOVA) with Fisher's Least

Significant Difference (LSD) post hoc test in MS excel, when there is one independent variable.⁴⁸In case of two independent variables, two-way ANOVA analysis with replication was performed. The Least Significant Difference (LSD) between any two groups a and b with a number of observations N_a and N_b , respectively, provided the ANOVA F omnibus is significant, was calculated by using the following formula:

$$LSD = t_{v,\alpha} \sqrt{MSW \left(\frac{1}{N_a} + \frac{1}{N_b} \right)}$$

Where MSW denotes the mean square of error within group and $t_{v,\alpha}$ is the value of t statistics at level of significance α with degrees of freedom v . The statistical analysis was performed at significance level $\alpha=0.05$ and 0.01 and presented as whichever is applicable.

3. Results and Discussion

3.1 Electron microscopy

Scanning electron micrographs confirm the formation of nanofibres of MEH-PPV:PCL by a simple electrospinning method (Figure 2). The electrospun meshes obtained by varying MEH-PPV to PCL volume ratios of 20:80, 40:60, 50:50 and 60:40 were named as SEN1, SEN2, SEN3 and SEN4, respectively. The core-sheath fibres electrospun at a flow rate of 0.6 mL/h and 1mL/h were denoted as CSN1 and CSN2. The diameter of nanofibres produced by simple electrospinning method varies with the variation of the volume ratio of MEH-PPV to PCL. SEN1, having the lowest volume percent of MEH-PPV to PCL (20:80 v/v) has the largest diameter of 324 ± 70 nm. The diameters of SEN2 (40:60 v/v), SEN3 (50:50 v/v) and SEN4 (60:40) have been measured to be 280 ± 82 nm, 198 ± 30 nm and 132 ± 53 nm using Carl Zeiss Software. The scanning electron micrographs of electrospun nanofibres prepared by a simple electrospinning process further confirm an increase in bead formation with an increase in MEH-PPV concentration due to its poor electrospinning ability. However, SEN1 and core-sheath nanofibres are free from beaded fibres. It is believed that the poor electrospinning

ability of MEH-PPV, may be the reason for the decrease in fibre diameter with increase in MEH-PPV concentration.^{49,50} The nanofibres produced by a core-sheath electrospinning method have been found to possess a larger diameter than the nanofibres produced by a simple electrospinning method as shown in Figure 3. CSN1 [Figure 3 (a1 & a2)], electrospun at a flow rate of 0.6 mL/h, has a diameter of 526 ± 60 nm, whereas CSN2 [Figure 3 (b1 & b2)], electrospun at flow rate of 1 mL/h, has the fibre diameter of 630 ± 137 nm. The core-sheath morphology of CSN1 and CSN2 has been confirmed by transmission electron micrographs c1 and c2 in Figure 4, respectively. The core (PCL) thickness of CSN1 and CSN2 has been measured to be 255 ± 62 nm and 409 ± 91 nm, respectively, from TEM images using ImageJ software. These results are consistent with earlier reports that higher flow rate produces nanofibres of larger diameter.^{51,52}

3.2 Current- voltage (*I-V*) characteristics and surface resistance

Figure 4 (a & b) show the room temperature (300 K) nonlinear *I-V* characteristics of electrospun nanofibres produced by a simple electrospinning and coaxial electrospinning process, respectively. The *I-V* characteristics show nonlinear behavior which is quite symmetric with respect to polarity in the applied voltage range of -10 V to +10 V. In case of the electrospun nanofibres prepared from the blend of MEH-PPV and PCL, the value of current is higher at a particular voltage for higher concentration of FeCl₃ doped MEH-PPV. The core-sheath nanofibres show improved *I-V* characteristics with a higher value of current when compared to the nanofibres prepared from the blend of MEH-PPV and PCL by a simple electrospinning process. The higher concentration of charge carriers with increasing concentration of FeCl₃ doped MEH-PPV results in a higher current value as observed in the *I-V* characteristics of the nanofibres prepared by a simple electrospinning process. FeCl₃ doped MEH-PPV on the surface of the core-sheath nanofibres also contributed towards the improved *I-V* characteristics. The log-log plot of the positive side of the corresponding current-

voltage (I-V) data can provide better insight into the conductive mechanisms in the different electrospun nanofibres.⁵³ The log-log plots show two distinct regions with a gradual transition between the two regions: one in the lower voltage region ($0 < V < 3$ for SEN1 and SEN2; $0 < V < 2$ for SEN3, SEN4, CSN1 and CSN2) and the other in the higher voltage region ($3 < V < 10$) [Figure 4 (c-h)]. These two distinct linear regions on the log-log plot can be fitted to a power law equation with different exponents, expressed as:

$$I = KV^m$$

where K is a constant and m is the exponent, which can be obtained from the slope of the fitted curve. In the lower voltage region, the exponent (m_1) is nearly unity and in the higher voltage region, the exponent (m_2) is different from unity as shown in Figure 4(c-h). It indicates that at lower voltage region, the current varies linearly with voltage suggesting the charge transport mechanism is Ohmic, whereas current varies non-linearly in the higher voltage region suggesting space charge limited conduction (SCLC). The observed I-V characteristics, with two power law regions are consistent with the space-charge limited conduction (SCLC) due to the presence of trapped charges in MEH-PPV.⁵⁴ At low voltages, the number of injected electrons is very small as compared to the intrinsic carriers making the charge transport mechanism ohmic. As the bias voltage is increased above 2-3 V, a transition from Ohmic to non-Ohmic behaviour takes place, when the density of the injected carriers becomes comparable to the density of the thermally generated free carriers and SCLC occurs. The bias voltage at which the transition from Ohmic to non-Ohmic behaviour occurs is called the critical voltage (V_c) and can be expressed as follows:⁵⁵

$$V_c = \frac{8qp_0d^2}{9\varepsilon_0\varepsilon_r\theta}$$

where p_0 is the density of thermally generated charge carriers, d is the sample thickness, ε_0 the permittivity in free space and ε_r is the dielectric constant of the sample. The trap factor is given by, $\theta = p/(p+p_t)$, where p is the density of free charge carriers and p_t is the density of

trapped charge carriers, increases due to increase in the free charge carrier density (p) in the sample. The critical voltages (V_c) determined from the log-log plot from the intersection of the two linear lines extended from the linear fit as shown in Figure 4 (c-h) shift towards the lower voltage side with increasing MEH-PPV concentration for nanofibres prepared from the simple electrospinning process (Table I). The core-sheath nanofibres also have lower critical voltages (V_c) (Table I). The lower values of V_c for the core-sheath nanofibres (CSN1 & CSN2) and nanofibres with higher MEH-PPV concentration (SEN4<SEN3<SEN2<SEN1) are assigned to the higher density of free charge carriers (p) due to FeCl₃ doped MEH-PPV, which results in improved I-V characteristics (CSN2>CSN1>SEN4>SEN3>SEN2>SEN1) [Figure 4 (a & b)]. The results are further supported by the surface resistance (R_s) values of different electrospun nanofibres (Table I), which have been calculated using the formula given below:²⁷

$$\text{Surface resistance } (R_s) = R \times \frac{W}{D}$$

Where W is the sample width and D is the distance between the two probes of the source metre. R is determined from the inverse of the slope of the I-V characteristics. The surface resistance of electrospun nanofibres produced by the simple electrospinning method decreases with increasing MEH-PPV concentration (Table I). The decrease in surface resistance with increase in MEH-PPV concentration, in turn, implies increase in surface conductivity of the electrospun nanofibres. With the highest concentration of conducting polymer (MEH-PPV) doped with FeCl₃, SEN4 was found to have the lowest surface resistance among the nanofibres prepared by the simple electrospinning process. This proposition has been further supported by lower surface resistance of core-sheath nanofibres, where the sheath material is conductive MEH-PPV with non-conductive PCL in the core (Table I).

3.3 Stability test

All the electrospun nanofibres were kept in PBS (pH =7.4) for 45 days to check stability in physiological solution. The electrospun nanofibres were characterized using SEM after 45 days to confirm that no degradation occurs and micrographs are presented in Figure 5. The fibrillar diameter was constant over time. The diameters of the nanofibres kept in PBS for 45 days did not decrease in comparison to those not kept in PBS. The diameters of SEN1, SEN2, SEN3, SEN4, CSN1 and CSN2 kept in PBS for 45 days have been measured to be 304 ± 71 , 272 ± 83 , 211 ± 30 , 133 ± 23 , 544 ± 208 and 617 ± 140 nm, respectively. To study the conductive properties, the surface resistivity of all the nanofibres kept in PBS for 45 days, was measured. The surface resistivity of these nanofibres was also not significantly different from their counterparts without treatment with PBS. The surface resistivity values of SEN1, SEN2, SEN3, SEN4, CSN1 and CSN2 have been determined to be $6.52 \pm 4.88 \times 10^7$, $4.24 \pm 3.05 \times 10^7$, $2.43 \pm 1.23 \times 10^7$, $8.68 \pm 2.76 \times 10^6$, $2.98 \pm 2.21 \times 10^5$ and $2.51 \pm 1.84 \times 10^5 \Omega$, respectively. The electrospun nanofibres have been found to be stable enough in physiological solution due to the non-degradable nature of MEH-PPV and slow degradation rate of PCL,⁴¹ which indicates the potential of these nanofibres as a conductive scaffold for neural tissue engineering.

3.4 FTIR spectroscopy

Electrospun nanofibres produced by both a simple electrospinning process and a coaxial electrospinning process display characteristic vibrational bands for both MEH-PPV and PCL such as C=C stretch (1677 cm^{-1}), C-C ring stretch ($1501\text{-}1598 \text{ cm}^{-1}$), aryl-alkyl ether (C-O-C) asymmetric stretch (1251 cm^{-1}) in MEH-PPV^{56,57} and similarly, C=O stretching of ester groups (1723 cm^{-1}), C-O and C-C stretching (1297 cm^{-1}), O-H stretching ($3500\text{-}3600 \text{ cm}^{-1}$) in PCL^{58,59} as shown in Figure 6 (a). The weak bending vibration in the range $729\text{-}735 \text{ cm}^{-1}$ is attributed to rocking motion of CH_2 groups present in aliphatic chain of MEH-PPV and

PCL. In particular, the prominent but weak triplet in the 1500-1700 cm^{-1} region corresponding to C=C stretch of paraphenylene vinylene (PPV) ring in FTIR spectra of CSN1 and CSN2, indirectly indicates more exposure of MEH-PPV to IR.⁶⁰ The highest energy of these modes is not IR active on a symmetric ring and only very weakly active in MEH-PPV.⁵⁷ This observation indirectly indicates the presence of MEH-PPV in the sheath of the core-sheath nanofibres.

3.5 X-ray photoelectron spectroscopy

XPS analysis was carried out in order to understand the surface chemistry of the electrospun nanofibres. Analysis of surface elemental composition by XPS survey scans suggest that carbon (C1s) and oxygen (O1s) were major composition of the electrospun nanofibres, with trace amounts of silicon (Si2p) and calcium (Ca2p) as external contaminants [Figure 6 (b)]. A small amount of chlorine Cl2p has been marked in SEN2, SEN4, CSN1 and CSN2, which indicates the doping level in MEH-PPV by FeCl_3 . Interestingly, the core-sheath nanofibres CSN1 (1.10%) and CSN2 (2.10%) contain a higher atomic percent of Cl2p than that present in SEN2 (0.40%) and SEN4 (0.50%), whereas it is negligible in SEN1 and SEN3. MEH-PPV present in the sheath of the core-sheath nanofibres is the reason for the higher doping level detected. The details of peak deconvolution of the C1s core-level spectra of SEN1 and CSN1 [Figure 6 (c & d)] are presented in Table II showing the presence of different chemical groups in MEH-PPV and PCL.

The C1s core-level spectrum of SEN1 can be deconvoluted into four peak components with binding energies (BEs) at about 284.99, 286.35, 287.79, and 288.96 eV, corresponding to C-C/C-H, C-OH/C-O, C=O and O=C-O species, respectively [Figure 6 (c) & Table II].⁶¹⁻⁶⁶ Similarly, the C1s core-level XPS spectrum of CSN1 contains four characteristics peaks at about 285.01, 286.11, 287.71, and 289.04 eV corresponding to C-C/C-H, C-OH/C-O, and C=O species, respectively [Figure 6 (d) & Table II]. However, the atomic% of C=O in CSN1

is relatively less than those in SEN1. The O=C-O species is ascribed to the ester groups of PCL, whereas the species like C-C/C-H, C-OH/C-O are also the common constituents in PCL.^{67,68} The C-C/C-H and C-O are the major components in MEH-PPV as reported earlier.⁶⁴ The C1s core-level XPS spectrum of CSN1 further demonstrates that there are no XPS peak for O-C=O species corresponding to ester groups of PCL, which confirms the lone presence of MEH-PPV on the sheath of the core-sheath nanofibres (Table II). Concurrently, the C1s core-level XPS spectrum of CSN1 is dominated by the peak at about 285 eV, which is characteristic of the neutral carbon species, indicating the presence of MEH-PPV in the sheath.⁶⁹ It is important to note that the C=O species is potentially a contamination of amide in DMF left out after electrospinning. DMF was used as a solvent during electrospinning of MEH-PPV:PCL blend. Although, the electrospun meshes were air dried for 24 h in order to evaporate the solvents, DMF may still be there since it is generally used as a solvent of low evaporation rate.⁷⁰ The samples were cleaned with rigorous washing with alcohol/water solution and deionized water prior to XPS characterisation but the cleaning process may not have removed DMF. DMF can be hydrolyzed only by strong acids and bases, especially at elevated temperatures.⁷⁰ This suggests that the C=O species, seen on all of the electrospun meshes, arises from the amide of DMF only.

3.6 Cell viability assay

MEH-PPV has never been tested for cytotoxicity on direct contact with mammalian cells, although Filipa Pires *et al.* reported the non-cytotoxic effect of extracts of MEH-PPV with L929 fibroblasts cells.³⁵ The MTS proliferation assay was performed using 3T3 fibroblasts and PC12 cells in direct contact with the electrospun meshes including pure MEH-PPV and PCL to investigate the cytotoxic effect of these materials on mammalian cells after incubation for 24, 48 and 72 h. The results suggest that all the electrospun meshes support cell viability of nearly 80% or above following direct contact for upto 72 h [Figure 7 (a) & (b)] indicating

their non cytotoxic behaviour with both types of cell line except for pure MEH-PPV (70-80% with both 3T3 & PC12 cells).⁷¹ The pure MEH-PPV demonstrated approximately 70% cell viability in contact with 3T3 and PC12 cells indicating slightly less cytocompatibility from the accepted cell viability level of 80% for biocompatible materials [Figure 7 (a) & (b)].⁷¹ Intriguingly, when blended with PCL, the cytotoxic effect was reduced significantly ($p < 0.01$) for all of the electrospun meshes as compared to pure MEH-PPV. This observation was consistent for all three time points. Similar observation applies to the core-sheath nanofibres as well, although core-sheath nanofibres had MEH-PPV as sheath material. However, the improved cell viability may be attributed to the nanofibrillar structure which was not present in pure MEH-PPV, and may mimic the ECM environment *in vitro* for the cells. After 72 h, cell viabilities for both 3T3 & PC12 cells on the electrospun meshes were comparable to those on the negative control, [Figure 7 (a) & (b)]. Moreover, no significant difference was observed in cell viability (both 3T3 & PC12 cells) with increasing concentration of MEH-PPV in electrospun meshes prepared by a simple electrospinning process or even in the core-sheath nanofibres, at any time points up to 72 hrs supporting the biocompatibility of the materials.

The long-term biocompatibility of the materials was assessed using a live/dead assay in order to visualize the viable 3T3 cells and PC12 cells on different electrospun meshes including the negative control (TCP) after 7 day of culture. The live/dead results with 3T3 fibroblasts demonstrate the calcein AM stained viable cells, which achieved more than 80% confluence, with very less number of EthD-1 stained dead cells [Figure S4 in ESI]. Similarly, the calcein AM stained viable PC12 cells are also clearly visible on all the electrospun meshes with few dead cells indicating their long-term compatibility with PC12 cells [Figure S5 in ESI]. Figure S5 demonstrates that collagen coated SEN1, SEN2, SEN3, CSN1 and CSN2 exhibit almost comparable cell viability (green) to the control (collagen coated coverslip). However, although not quantified, it appears that the electrospun meshes

(particularly, CSN1 & CSN2 followed by SEN1, SEN2 & SEN3) with larger diameter nanofibres exhibit significantly better cell attachment and subsequently, the viable cell numbers after 7 days of culture were also greater. Fewer cells attached on SEN4. However, the PC12 cells once attached on the scaffolds were viable and able to maintain normal cellular function in differentiating media including morphological changes such as neurite formation (discussed in detail in Section 3.7). The results of the live-dead staining assay support the MTS data indicating the biocompatibility of the MEH-PPV based electrospun meshes.

3.7 Beta (III) tubulin immunocytochemistry

The PC12 cells were cultured for 7 days in differentiating medium on different electrospun MEH-PPV:PCL meshes with and without collagen I coating to investigate the suitability of these materials for neuronal applications. This cell line has been widely used as a model neuronal system. PC12 cells cultured with NGF develop long neurite outgrowth, become electrically excitable and take on many of the biochemical traits of sympathetic noradrenergic neurons.⁷² Since, PC12 cells readily adhere to collagen, all the electrospun meshes were coated with collagen I. Collagen I is a fibril forming collagen present in the ECM of the peripheral nervous system (PNS) and plays an important part in the development of the peripheral nervous system as well as in the maintenance of normal peripheral nerve function during adulthood.⁷³ Immunolabelling with beta (III) tubulin antibody was performed to confirm PC12 cell differentiation into sympathetic neurons. The PC12 cells grown on the uncoated and coated electrospun meshes were labelled with beta (III) tubulin antibody to visualize cytoskeletal microtubules, which are dynamic polymer filaments of alpha and beta tubulin subunits that drive neurite outgrowth and control neuronal morphology.⁴⁷

Adherent cell number was counted as the total number of cells in 3 fields of view for each of three repeat samples per mesh type and the results indicate significantly improved cell adhesion to the collagen coated electrospun meshes with larger diameter nanofibres when

compared to the uncoated meshes ($p < 0.01$). Cell numbers counted on uncoated nanofibre mats of SEN1, SEN2, SEN3, SEN4, CSN1, and CSN2 are 24 ± 3 , 21 ± 10 , 12 ± 12 , 17 ± 8 , 43 ± 24 and 42 ± 17 (mean $\pm$ SEM) per substrate, respectively [Figure 8 (a1-f1)]. Electrospun nanofibre mats coated with collagen display improved cell adhesion with cell numbers of 185 ± 26 , 181 ± 31 , 184 ± 45 , 144 ± 37 , 194 ± 23 and 203 ± 54 per substrate on SEN1, SEN2, SEN3, SEN4, CSN1 and CSN2, respectively [Figure 8 (a2-f2)]. The collagen coated cover slip control has 158 ± 59 cells per image indicating that the collagen coated mats showed cell adhesion equivalent to that of the control [Inset of Figure 8 (g)]. It was also observed that the collagen coated blended nanofibres with larger fibre diameter, i.e., SEN1, SEN2 and SEN3 demonstrate significantly higher PC12 cell attachment when compared to that on SEN4 with the smallest nanofibre diameter [Figure 8 (a2-d2), $p < 0.01$]. A similar statistically significant difference exists between the collagen coated core-sheath nanofibres having larger fibre diameter and the blended nanofibres having relatively smaller diameter than the former [Figure 8 (a2-f2), $p < 0.01$]. This observation is in well agreement with the report that larger diameter fibres favour better cell attachment.⁷⁴ Nonetheless, there are no statistical differences in cell density between coated SEN1 *vs* SEN2 ($p = 0.90$), SEN1 *vs* SEN3 ($p = 0.62$), SEN2 *vs* SEN3 ($p = 0.66$) and CSN1 *vs* CSN2 ($p = 0.78$).

The beta (III) tubulin protein staining confirms the neural differentiation of the PC12 cells. The tubulin staining can be visualized all through the cell bodies and neurites formed on uncoated electrospun meshes [Figure 8 (a1-f1)] and coated electrospun meshes [Figure 8 (a2-f2)]. Beta (III) tubulin labeling indicates the consistent neuronal morphology for the differentiated PC12 cells on the coated electrospun meshes and glass cover slips as compared to the uncoated electrospun meshes. The majority of the differentiated PC12 cells on the coated electrospun meshes have the neuronal characteristics including long neurites with or without branches of varying complexity, round somas of variable size and many growth cones

[Figure 8 (a2-f2)]. In contrast, the cells on the uncoated meshes form clusters with very poor or short neurite formation with few or no branches and growth cones [Figure 8 (a1-f1)]. Measurement of neurite bearing cells and neurite outgrowth using ImageJ software indicated a significant increase in the number and length of neurites on collagen coated electrospun meshes in comparison to the uncoated meshes [Figure 8 (g & h), $p < 0.01$]. At least 600 cells and 250 neurites underwent quantitative analysis of neurite formation and neurite outgrowth across 3 fields of view per coated electrospun mesh in triplicate. Due to poor attachment of PC12 cells on uncoated electrospun meshes, the number of cells available for analysis was below 150 per substrate.

The number of differentiated PC12 cells that formed neurites on the coated core-sheath nanofibres, i.e., CSN1 ($43 \pm 14\%$, $N=542$), CSN2 ($46 \pm 21\%$, $N=628$) were significantly higher ($p < 0.01$) than that on the coated blended nanofibres, i.e., SEN1 ($41 \pm 9\%$, $N=557$), SEN2 ($31 \pm 12\%$, $N=524$), SEN3 ($33 \pm 7\%$, $N=489$) and SEN4 ($30 \pm 10\%$, $N=494$), where N is number of cells analysed across 3 fields of view per mesh in triplicate [Figure 8 (g)]. Moreover, the number of neurite bearing cells on the coated SEN1 has been also found to be statistically different from that on the coated SEN2, SEN3 and SEN4 at $p < 0.01$. Yet, the statistical difference does not exist between SEN2 vs SEN3 ($p=0.80$), SEN2 vs SEN4 ($p=0.50$), SEN3 vs SEN4 ($p=0.82$) and CSN1 vs CSN2 ($p=0.30$). The neurite length per cell on coated electrospun meshes of CSN1 ($93 \pm 14 \mu\text{m}$, $m=346$), CSN2 ($97 \pm 24 \mu\text{m}$, $m=324$) and SEN1 ($96 \pm 16 \mu\text{m}$, $m=332$) is statistically different ($p < 0.01$) from that on the coated electrospun meshes of SEN2 ($83 \pm 11 \mu\text{m}$, $m=284$), SEN3 ($82 \pm 20 \mu\text{m}$, $m=265$) and SEN4 ($78 \pm 12 \mu\text{m}$, $m=271$), where m denotes the number of neurites analyzed [Figure 8 (h)]. Unlike the cell density and the neurite bearing cells, the neurite length on the core-sheath nanofibres has not been found to be statistically different from that on the coated SEN1. Besides, there are no significance differences in neurite length per cell between SEN2 vs

SEN3 ($p=0.81$), SEN2 *vs* SEN4 ($p=0.60$), SEN3 *vs* SEN4 ($p=0.93$) and CSN1 *vs* CSN2 ($p=0.51$). These results indicate that electrospun meshes CSN1, CSN2 and SEN1 with nanofibres of larger fibre diameter favoured more neurite formation as well as longer neurite outgrowth than those on SEN2, SEN3 and SEN4 with nanofibres of comparatively smaller diameter ($p<0.01$).

To check whether only collagen coating contributed mostly towards PC12 adhesion, its neural differentiation and subsequent neurite outgrowth or not, the PC12 cells were also cultured on collagen coated glass cover slips and subjected to the differentiation protocol. The cell counts, the percentage of neurite bearing cells and the neurite length per cell on the collagen coated glass cover slip were 174 ± 26 , 38 ± 12 ($N=444$) and 75 ± 18 ($m=271$) μm , respectively and were significantly lower than those on CSN1, CSN2 and SEN1 ($p<0.01$). The results suggest that although the collagen coating has the major role in PC12 adhesion and differentiation, the material and its morphology have also some impact on PC12 behaviour on these scaffolds. In short, the fibre features and diameter, as well as collagen coating, played an important role in neurite formation and outgrowth on the electrospun meshes, which is consistent with previous reports.²⁷ It has been also noted that MEH-PPV in the sheath of the core-sheath nanofibres has no adverse effect on neurite formation and outgrowth, which is in agreement with the biocompatibility of MEH-PPV in the blended and core-shell formulations shown in Figure 7 and Figure S4 and S5.

3.8 Cell adhesion study using scanning electron microscopy

To further confirm the attachment of PC12 cells and to study their morphology on various electrospun meshes, SEM was performed with the same samples used for immunostaining (Figure 9). The SEM images confirm the results of the immunocytochemistry. All of the electrospun meshes coated with collagen I display better cell adhesion and morphology in comparison to the poor cell adhesion on the uncoated electrospun. PC12 cells on coated

electrospun meshes appear to make contact with multiple fibres and form more elliptical morphologies with neurite projections [marked in red arrows in Figure 9 (a2-f2)]. The cells on the uncoated electrospun meshes have a spherical, [Figure 9 (a1-f1)] distorted morphology with few or no neurite projections. The results confirm significantly greater cell attachment on the coated electrospun meshes with the presence of neurite projections in random directions indicating the potential of the coated electrospun meshes over the uncoated meshes for electrical stimulation of PC12 cells.

3.9 Electrical stimulation of PC12 cell

The beta (III) tubulin immunochemistry results discussed in Section 3.7 demonstrate the ability to support the differentiation of the PC12 cells on the different electrospun meshes. To explore the potential of electrical stimulation of nerve cells through these conductive electrospun meshes as a scaffold for axonal regeneration in damaged nerves, electrical stimulation of differentiated PC12 cells was carried out. Electrospun meshes coated with collagen I were used as a conductive scaffold for electrical stimulation as they show better neurite formation and outgrowth than the uncoated meshes. The effect of electrical stimulation of PC12 cells through the conductive electrospun meshes has been assessed in terms of quantitative analysis of percentage of neurite bearing cells, neurite per cell, neurite length per cell and median neurite lengths from the confocal images of beta tubulin stained PC12 cells on electrospun meshes [Figure 10 (a2-f2)]. PC12 cells were also cultured on electrospun meshes in a bespoke electrical stimulation set up [Figure S2 (a)] without electrical stimulation for comparison, and representative confocal images are shown in Figure 10 (a1-f1). The results indicate that the presence of MEH-PPV in the blend and core-shell formulations increases the % neurite bearing cells and the neurite length per cell following 2 hrs growth under electrical stimulation. The most significant effects were when MEH-PPV was used in the core-sheath electrospun meshes as the sheath material for the

electrical stimulation, owing to the increased electrical conductivity. Healthy neuronal characteristics of differentiated PC12 cells including greater neurite formation, branching, and longer axonal growth were observed on electrically stimulated cells [Figure 10 (a2-f2)] as compared to unstimulated cells [Figure 10 (a1-f1)].

Quantitative analysis indicates the formation of more neurites in the electrically stimulated PC12 cells on SEN1 ($46 \pm 10\%$, $N=473$, $m=324$), SEN2 ($45 \pm 13\%$, $N=445$, $m=394$), SEN3 ($51 \pm 5\%$, $N=409$, $m=341$), SEN4 ($57 \pm 23\%$, $N=438$, $m=378$), CSN1 ($66 \pm 11\%$, $N=545$, $m=590$) and CSN2 ($64 \pm 10\%$, $N=476$, $m=514$) than the unstimulated PC12 cells on SEN1 ($38 \pm 11\%$, $N=481$, $m=232$), SEN2 ($33 \pm 9\%$, $N=461$, $m=203$), SEN3 ($31 \pm 5\%$, $N=519$, $m=219$), SEN4 ($32 \pm 7\%$, $N=502$, $m=222$), CSN1 ($44 \pm 8\%$, $N=604$, $m=450$) and CSN2 ($43 \pm 15\%$, $N=566$, $m=331$), where N and m denote number of cells and number of neurites analyzed [Figure 11 (g)]. The results indicate that electrical stimulation of PC12 cells has a statistically significant effect on neural differentiation. The percentage of neurite bearing cells on SEN2, SEN3, SEN4, CSN1, and CSN2 under electrical stimulation was significantly greater than those without electrical stimulation ($p < 0.01$), while it is statistically different at $p < 0.05$ in case of SEN1 as indicated in Figure 10 (g). Analysis of neurites per cell indicated that single stimulated cell had 1.29 (SEN1), 1.67 (SEN2), 1.64 (SEN3), 2.22 (SEN4), 2.12 (CSN1) and 2.6 (CSN2) neurites on average as compared to 1.33 (SEN1), 1.23 (SEN2), 1.17 (SEN3), 1.18 (SEN4), 1.42 (CSN1) and 1.34 (CSN2) neurites per unstimulated cells [Figure 10 (h)], which are statistically significant at $p < 0.01$ except SEN1. Quantitative analysis to determine the effect of electric field on axonal growth reveals that the neurite lengths per cell and median neurite lengths of electrically stimulated cells are longer on SEN1 (119 ± 12 and $67 \pm 7 \mu\text{m}$), SEN2 (148 ± 43 and $72 \pm 6 \mu\text{m}$), SEN3 (137 ± 21 and $73 \pm 11 \mu\text{m}$), SEN4 (172 ± 70 and $75 \pm 8 \mu\text{m}$), CSN1 (166 ± 42 and $79 \pm 7 \mu\text{m}$) and CSN2 (182 ± 68 and $83 \pm 21 \mu\text{m}$) than those of the unstimulated cells on SEN1 (90 ± 19 and $59 \pm 9 \mu\text{m}$), SEN2 (76 ± 15 and 51

$\pm 7 \mu\text{m}$), SEN3 (73 ± 18 and $53 \pm 5 \mu\text{m}$), SEN4 (69 ± 26 and $44 \pm 4 \mu\text{m}$), CSN1 (95 ± 22 and $55 \pm 7 \mu\text{m}$) and CSN2 (89 ± 25 and $57 \pm 13 \mu\text{m}$) [Figure 10 (i) & (j)] and these electrical stimulation results were significant at $p < 0.01$.

Any statistical differences in the percentage of neurite bearing cells, neurite per cell, neurite length per cell and median neurite length among the various the electrospun meshes have been indicated in Figure 10 (g-j). It was observed that under electrical stimulation, the core-sheath nanofibres (CSN1 & CSN2) and the blended nanofibres, particularly, SEN4 demonstrated significantly greater neurite formation and longer neurite outgrowth on them than those on the SEN1, SEN2 and SEN3. The detailed statistical analysis of neurite formation and neurite outgrowth on all the electrospun meshes under electrical stimulation has been provided in the ESI.

The results suggest that electrical stimulation of PC12 through the electrically conductive electrospun meshes contributes positively towards both neurite formation and outgrowth. These observations reveal that electrospun meshes having lower surface resistance and higher density of free charge carriers (particularly, CSN1, CSN2 & SEN4) displays more neurite formation and longer neurite outgrowth under electrical stimulation. From the statistical analysis of neurite formation data, it can be stated that the enhancement in neurite bearing cells and neurite per cell are more significant ($\alpha=0.01$) for the electrospun meshes except SEN1, having the highest sheet resistance and the lowest density of free charge carriers. The variation of the observed neurite characteristics under electrical stimulation according to the conductive properties of the electrospun meshes have been summarized in Table III.

The parameters to define the degree of neurite formation and outgrowth such as neurite bearing cells, neurite length per cell, median neurite length and neurite per cell under electrical stimulation increased with increasing concentration of conductive MEH-PPV in the case of the blended nanofibres produced by simple electrospinning process. The statistical

analysis suggests that in the case of the blended nanofibres, all these parameters are significantly enhanced on SEN4, when compared to the same parameters on SEN1 and SEN2. SEN4 owing to its higher MEH-PPV content possesses lower surface resistance and higher free charge carrier density, whereas SEN1 and SEN2 due to lower MEH-PPV content in them, possess higher surface resistance and lower free charge carrier density. The data analysis indicates that the core-sheath nanofibres owing to their lowest surface resistance and the highest free charge carrier density, demonstrate significant enhancement in percentage of neurite bearing cells, neurite per cell, neurite length per cell and median neurite length when compared to the blended nanofibres prepared by simple electrospinning process.

These observation reveals the major role of the conductive polymer in the neurite formation and outgrowth under electrical stimulation. It is important to note that although under no electrical stimulation, the collagen coating and the diameter of the nanofibres played the critical role in modulating the neurite formation and outgrowth [Section 3.7], the electrical stimulation through those same scaffolds has been demonstrated to significantly enhance the same along with the positive contribution from the scaffold morphology and collagen coating. In addition, to evaluate the synergistic effect of the scaffold and electrical stimulation, the same electrical stimulation experiments were repeated without NGF, which plays a major role in the neuronal differentiation of PC12 cells. Interestingly, the PC12 cells differentiated to their neuronal phenotype under electrical stimulation through the electrospun meshes as demonstrated by beta (III) tubulin immunostaining after 7 days [Figure S6]. This observation is also in agreement with previous studies by Kimura *et al.*, where PC12 cell differentiation to neuronal phenotype was demonstrated due to electrical stimulation induced calcium influx and *c-fos* mRNA expression.⁴⁶ However, the percentage of the neurite bearing cells were measured to be $13 \pm 8\%$ (SEN1), $16 \pm 6\%$ (SEN2), $15 \pm 4\%$ (SEN3), $21 \pm 8\%$ (SEN4), $23 \pm 6\%$ (CSN1), and $24 \pm 9\%$ (CSN2), which are relatively less when compared to the results in the presence of NGF.

The current signal applied by a chronoamperometric method in pulse mode for stimulation of PC12 cells through different electrospun meshes is shown in Figure S7. The degree of neurite formation and outgrowth increases almost systematically with increase in current signal (upto 3-4 μA) through the conductive electrospun scaffolds during stimulation (CSN2>CSN1>SEN4>SEN3.SEN2>SEN2>SEN1) [Table III]. Our finding is in agreement with that reported by Zhang *et al.* More neurite-bearing PC12 cells were observed below 10 μA and promotion of neurite formation diminished as currents increased above 10 μA .²⁷ Under the application of ES, the availability of free charge carriers in the conductive electrospun meshes (more in CSN1, CSN2 and SEN4) resulted in more charge-transport between the scaffold and the cell membrane. This charge-transport process changes the resting membrane potential of differentiated PC12 cells. Under constant electrical potential for 2 h, the cell membrane undergoes an intensity-dependent Depolarisationresulting action potential, which is responsible for axonal growth. This change in cell membrane potential is also believed to activate growth-controlling transport processes across the plasma membrane and cause redistribution of cytoplasmic materials.⁷⁵⁻⁷⁷ It has been shown that membrane Depolarisationinduces neurite outgrowth due to an elevated concentration of K^+ .⁷⁸ The elevated concentration of K^+ is further believed to induce immediate early genes (IEGs), that are responsible for Depolarisationof neuronal cells⁷⁹ and differentiation.⁸⁰ Cavalie *et. al* showed that extracellular calcium influx is required to induce IEGs by depolarization.⁸¹ Kimura *et. al* showed that electrically induced *c-fos* mRNA expression due to calcium influx via an L-type calcium ion channel causes differentiation of PC12 cells without NGF.⁴⁶ According to Patel *et al.*, ES causes electrophoretic accumulation of surface molecules on the working electrode (scaffold).⁷⁵ All these are likely to be responsible for longer neurite growth or cell-substratum adhesion. However, the exact mechanism for the observed effect in this study is unclear and is a subject of future investigation.

4. Conclusion

We have developed an electrically conductive, MEH-PPV:PCL nanofibrillar mesh in blended and core-shell form using an electrospinning technique and optimised a uniform synthesis route for neuronal regeneration. We have carried out physico-chemical and biological characterisation of the meshes and confirmed that increasing concentrations of MEH-PPV in a PCL blend improved biocompatibility of MEH-PPV alone, reduced nanofibrillar diameter and tensile strength but increased conductivity and subsequent differentiated neuronal growth characteristics on cell seeded collagen coated meshes under electrical stimulation. We have shown that a core-shell synthesis route with MEH-PPV shell increased fibrillar diameter and tensile strength characteristics whilst improving conductive growth stimulus characteristics for neurite outgrowth on collagen coated meshes. Coaxial electrospinning produces uniform nanofibres with larger diameters and better conductive and mechanical properties than the electrospun nanofibres produced by the simple electrospinning process. This was confirmed by SEM, TEM and tensile strength measurements [Figure S3]. XPS results support higher doping levels in the core-sheath nanofibres leading to better conductive properties and indirectly confirming MEH-PPV in the sheath. Biocompatibility of all electrospun nanofibres produced by simple or coaxial electrospinning was confirmed using an MTS proliferation assay with both fibroblast and neuronal cell lines. Direct cell contact assays were carried out with MEH-PPV for the first time and cell viability over 7 days was confirmed using live/dead staining. The results support the potential use of MEH-PPV based biomaterial scaffolds to fabricate nerve guidance channels and bridge the gap for directive growth of damaged nerves in the PNS. These scaffolds could act as an alternative to conventional nerve auto and allografts.. MEH-PPV also provides a new additional option using CPs in neural tissue engineering applications as an alternative to the widely investigated PPy, PANI and PEDOT. The poor solubility exhibited by these polymers inhibits nanofibril formation by electrospinning whereas MEH-PPV with PCL has been shown to produce nanofibrous

scaffolds with varying morphology for potential neuronal stimulation. Additionally, initial evidence for the neural differentiation of PC12 cells in the absence of NGF on these electrically conductive electrosun meshes provides scope for further investigation into the exact mechanism of electrically induced neurite formation and outgrowth using this model.

5. Conflicts of interest

There are no conflicts of interest to declare.

6. Acknowledgements

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Table I. Calculated surface resistance (R_s) values of the different electrospun meshes prepared by simple electrospinning and coaxial electrospinning process.

Sample name	Surface resistance (R_s) Ω	Critical voltage (V_c) V
SEN1	$5.27 \pm 1.73 \times 10^7$	2.81
SEN2	$3.17 \pm 1.03 \times 10^7$	2.39
SEN3	$1.52 \pm 0.93 \times 10^7$	1.90
SEN4	$7.45 \pm 2.02 \times 10^6$	1.33
CSN1	$1.94 \pm 1.15 \times 10^5$	1.13
CSN2	$1.35 \pm 0.65 \times 10^5$	1.01

Table II: Details of C1s narrow scan spectra of SEN1 (20:80 v/v) and CSN1 (0.6 mL/h) and corresponding C1s peak deconvolution.^{60, 62}

Chemical group	SEN1		CSN1	
	Peak B. E.	Atomic %	Peak B. E.	Atomic %
C-C, C-H	284.99	65.82	285.01	78.21
C-OH, C-O	286.35	16.59	286.11	17.02
C=O	287.29	5.03	287.71	4.25
O-C=O	288.96	12.56	-	-

Table III: Comparison between the conductive properties of the different electrospunmeshes along with the current flowing through them during stimulation and the electrically stimulated neurite formation and outgrowth.

Sample		Current (μA)	With electrical stimulation			
			%Neurite bearing cells	Neurite per cell	Neurite length per cell (μm)	Median neurite length (μm)
Increasing R_s ↑ SEN1 SEN2 SEN3 SEN4 CSN1 CSN2 ↓ Decreasing V_c	SEN1	0.25-0.3	46 ± 10%	1.29 ± 0.1	119 ± 12	67 ± 7
	SEN2	0.5-0.6	45 ± 13%	1.67 ± 0.35	148 ± 43	72 ± 6
	SEN3	1.3-1.4	51 ± 5%	1.64 ± 0.22	137 ± 21	73 ± 11
	SEN4	2.6-2.75	57 ± 23%	2.22 ± 0.7	172 ± 70	75 ± 8
	CSN1	2.7-2.9	66 ± 11%	2.12 ± 0.38	166 ± 42	79 ± 7
	CSN2	3.9-4.1	64 ± 10%	2.6 ± 0.75	182 ± 68	83 ± 21

Figure 1. Schematic illustration showing flowchart of the electrospinning of MEH-PPV:PCL by (a) simple electrospinning technique and (b) coaxial electrospinning technique, PC12 cell culture on collagen coated electrospun meshes and electrical stimulation.

Figure 2. Scanning electron micrographs of electrospun nanofibres prepared by simple electrospinning of blend of MEH-PPV and PCL at various volume ratios. a1 & a2: SEN1 (20:80), b1 & b2: SEN2 (40:60), c1 & c2: SEN3 (50:50) and d1 & d2: SEN4 (60:40). Suffix ‘1’ and ‘2’ stand for magnification at 5 K and 50 K, respectively. Scale bar: 4 μm (a1, b1, c1 & d1) and 400 nm (a2, b2, c2 & d2).

Figure 3. Scanning electron micrographs of CSN1 (a1 & a2) and CSN2 (b1 & b2), acquired at two different magnifications of 5 K and 50 K. Transmission electron micrographs of CSN1 (c1) and CSN2 (c2) showing the formation of nanofibres with core-sheath morphology. Scale bar = 4 μm (a1 & b1), 300 nm (a2 & b2) and 500 nm (c1 & c2).

Figure 4. I-V characteristics of electrospun nanofibres produced by (a) simple electrospinning and (b) co-axial electrospinning process. Plots of forward I-V data on a log-log scale for (c) SEN1, (d) SEN2, (e) SEN3, (f) SEN4, (g) CSN1 and (h) CSN2 at room temperature (300 K) showing the fitting parameters.

Figure 5. Scanning electron micrographs of (a) SEN1, (b) SEN2, (c) SEN3, (d) SEN4, (e) CSN1 and (f) CSN2, recorded after keeping in PBS (pH=7.4) for 45 days (Scale bar = 5 μ m).

Figure 6. (a) FTIR spectra of all electrospun meshes as indicated; (b) Surface elemental compositions (% atomic concentration) in electrospun meshes as determined by XPS; Peak deconvolution of high resolution C1s XPS spectra of (c) SEN1 and (d) CSN1.

Figure 7. Percentage cell viability on different electrospun meshes in direct contact after 24, 48 and 72 h of culture as compared to negative control [tissue culture plastic (TCP)] and positive control (dibutyltin maleate). Viability of (a) 3T3 fibroblasts and (b) PC12 cells expressed as percentage of the negative control. Data are expressed as Mean $\pm$ S.D, n=4. * and ** indicate statistically significant difference at $p < 0.05$ and $p < 0.01$, respectively.

Figure 8. Immunolabelling of beta (III) tubulin in differentiated PC12 cells cultured for 7 days on uncoated and collagen I coated electrospun meshes with DAPI stained nuclei (Scale bar = 75 μm). a1, b1, c1, d1, e1 and f1 depict representative confocal images with phase contrast overlay showing subordinated differentiation of PC12 cells on uncoated electrospun meshes of SEN1, SEN2, SEN3, SEN4, CSN1 and CSN2, respectively. a2, b2, c2, d2, e2 and f2 show representative confocal images of improved neurite formation and outgrowth in differentiated PC12 cells grown on coated electrospun meshes as mentioned above. White and yellow arrows show neuronal cell bodies and neurite formed, respectively. Red arrows

represent neurons with long branched neurites and/or growth cones. Percentage of neurite bearing cells (g) and neurite length per cell (h) on both uncoated and coated electrospun meshes along with collagen coated glass presented as Mean $\pm$ S.D. Inset of (g) shows confocal images of stained PC12 cells cultured on collagen coated cover slip for 7 days. ** indicates statistically significant difference at $p < 0.01$, respectively ($n=3$).

Figure 9. Scanning electron micrographs of PC12 cells after 7 days cultured on uncoated (a1-f1) and coated (a2-f2) electrospun meshes of SEN1, SEN2, SEN3, SEN4, CSN1, and CSN2. Red arrows point to the neurite projection. Scale bar = 5 μ m.

Figure 10. Confocal images with phase contrast overlay of beta (III) tubulin immunostained PC12 cells cultured for 7 days under no electrical stimulation (a1-SEN1, b1-SEN1, c1-SEN3,

d1-SEN4, e1-CSN1, f1-CSN2) and under electrical stimulation of 500 mV/cm for 2h/day (a2-SEN2, b2-SEN2, c2-SEN3, d2-SEN4, e2-CSN1, f2-CSN2) [Scale bar = 75 μ m]; (g) Percentage of neurite bearing cells, (h) Neurite per cell, (i) Neurite length per cell and (j) Median neurite length of differentiated PC12 cells on various electrospun meshes without electrical stimulation and with electrical stimulation. Data are expressed as Mean $\pm$ S.D. * and ** indicate statistically significant difference at $p < 0.05$ and $p < 0.01$, respectively (n=3).

Table of Contents

Electrically conductive, porous, mechanically strong and bioactive electrospun MEH-PPV:PCL nanofibres with blended and core-sheath formulations for enhanced neurite formation and neurite outgrowth.