Protocol and cell responses in three-dimensional conductive collagen gel scaffolds with conductive polymer nanofibres for tissue regeneration

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It has been established that nerves and skeletal muscles respond and communicate via electrical signals. In regenerative medicine, there is current emphasis on using conductive nanomaterials to enhance electrical conduction through tissue-engineered scaffolds to increase cell differentiation and tissue regeneration. We investigated the role of chemically synthesized polyaniline (PANI) and poly(3,4-ethylenedioxythiophene) (PEDOT) conductive polymer nanofibres for conductive gels. To mimic a naturally derived extracellular matrix for cell growth, type I collagen gels were reconstituted with conductive polymer nanofibres and cells. Cell viability and proliferation of PC-12 cells and human skeletal muscle cells on these three-dimensional conductive collagen gels were evaluated in vitro. PANI and PEDOT nanofibres were found to be cytocompatible with both cell types and the best results (i.e. cell growth and gel electrical conductivity) were obtained with a low concentration (0.5 wt%) of PANI. After 7 days of culture in the conductive gels, the densities of both cell types were similar and comparable to collagen positive controls. Moreover, PC-12 cells were found to differentiate in the conductive hydrogels without the addition of nerve growth factor or electrical stimulation better than collagen control. Importantly, electrical conductivity of the three-dimensional gel scaffolds increased by more than 400% compared with control. The increased conductivity and injectability of the cell-laden collagen gels to injury sites in order to create an electrically conductive extracellular matrix makes these biomaterials very conducive for the regeneration of tissues.

1. Introduction

Scaffolds play an important role in tissue regeneration. Engineering an optimal environment for cells to develop into specific tissues is critical for the long-term functionality of regenerated tissue. Cells live in a dynamic environment, and therefore the directed design of a tissue relies not only on the tissue scaffold composition and topography, but also on the ability to control physical factors, such as mechanical and electrical signals, in the developing tissue. This is especially important for certain cells and tissues, such as neurons, skeletal muscles and heart, which respond to pulsatile and abrupt electrical stimuli.

Directed tissue regeneration is necessary for the effective treatment of nerve injuries. Nerve injuries are among the primary causes of human disability, including lost mobility and sensory function [1]. Owing to their direct impact on quality of life, neural repair and regeneration have received increasing attention in the field of tissue engineering. While autologous nerve grafts currently serve as the gold standard for treating neural defects, they have several...
limitations, including the loss of function at the donor graft site and mismatch of damaged nerve and graft dimensions [2,3].

As nerves use bioelectricity during their signalling and functional maintenance, electrical stimulation is an attractive method to stimulate cell proliferation and differentiation for neural tissue regeneration. Conducting polymers are responsive to electrochemical or electrical signals. The incorporation of conductive polymers allows for localized and external control of electrical stimuli directly to the cells through the electrically conducting scaffolds in order to promote differentiation into nerves [4]. Various conducting polymers, including polyaniline (PANI) and poly(3,4-ethylenedioxythiophene) (PEDOT), have been used to provide synthetic-conducting environment for cell proliferation and neurite outgrowth [5,6]. Moreover, they are widely used for neural tissue engineering applications, such as neuronal interface [7], recording [8] and stimulations [9].

One potential approach is the fabrication of biomimicking extracellular matrix analogues, containing conductive polymers, such as PANI and PEDOT, which are suitable for implantation and can support three-dimensional cell culture. Scaffolds electrodeposited with PEDOT increase axon growth in nerve conduits [5]. Hybrid macroporous hydrogels made of PANI and poly(ethylene glycol) diacylate improve the differentiation of PC-12 and human mesenchymal stem cells [6]. Longer neurite extension and proliferation are observed on an electrically conductive substrate [2,10,11]. In addition, through a combination of tissue-engineered scaffold topography, electrical stimulation and biomolecule entrapment, directed cellular growth of nerve tissue regeneration can be controlled to improve neurite extension and nerve regeneration [1,4,12,13]. This approach may also be applicable to skeletal muscle, another electro-responsive tissue. For example, myoblasts increase myotube formation on electrically conductive scaffolds, even in the absence of electrical stimuli [14,15]. Extracellular cues influence myotube formation and muscle contraction on electrically conductive scaffolds in the presence of electrical stimulation [16–18]. Therefore, by incorporating conducting polymer nanofibres into the three-dimensional scaffolds, the electrical conduction of scaffolds may increase, while maintaining biocompatibility and supporting cell differentiation.

As the electrical conductivity of conductive polymers, such as PANI and PEDOT, is sensitive to the chemical structure of the polymer, it is difficult to directly functionalize polymers with biological moieties without negatively impacting the electrical conductivity. However, the presence of these biological groups is very important for supporting cell growth and differentiation. As a compromise, dispersing the electrically conductive polymer within a biological matrix may allow the formation of an electrically conductive biomaterial composite. Type I collagen is the most abundant natural protein in the extracellular matrix of various tissues (e.g. skin, tendon, bone, ligament, cornea and internal organs). Thus, collagen is a great candidate protein to serve as a naturally derived biomaterial to facilitate the regeneration of damaged tissues. Collagen provides good cell attachment sites via integrin-binding proteins and is generally non-immunogenic among various mammalian species when implanted in vivo [19]. The ability of collagen solution to gel at a neutral pH and physiological temperature allows collagen solution to be injected to fill lesions or implant cell-seeded gel scaffolds at the injured site [20]. An acetic acid-soluble collagen containing non-helical end regions undergoes in vitro fibrillogenesis in a nucleation phase into collagen fibrils with weak interactions that are subsequently enhanced by covalent cross-linking in a rapid growth phase [19,21]. In the cell-free environment, the fibrils are formed as a highly interconnected random network or mesh of long continuous fibrils with different D-shaped characteristic, length and diameter depending on the growth conditions (such as pH, temperature, buffer, ions and protein concentration), the source, type of collagen and extraction method [22,23]. In situ gelling scaffolds are particularly attractive for non-invasive approaches to tissue engineering and regeneration [24–26]. Therefore, dispersing a conductive polymer into an assembled collagen matrix may allow for the generation of an electrically conductive biomaterial.

In this study, two cell types, PC-12 cells and human skeletal muscle cells (hSMCs), were used as model systems to test cell viability and proliferation in our conductive gel scaffolds in vitro. To fabricate these scaffolds, two specific conductive polymers, PANI and PEDOT nanofibres, were chemically synthesized and added to the collagen solutions before mixing with the cell suspensions. The biocompatibility of the nanostructured, electrically conductive collagen gel scaffolds was studied by evaluating cell viability and proliferation using cell assay and confocal microscopy. To assess PC-12 cell differentiation, neurite outgrowth in the three-dimensional conductive collagen gel scaffolds was evaluated by immunofluorescence staining and western blot without the induction of nerve growth factor (NGF). The results of cell culture studies using various concentrations of PANI or PEDOT nanofibres were analysed and compared to determine the optimal composition of conductive collagen gel scaffolds for tissue engineering.

2. Materials and methods

2.1. Chemical synthesis of polyaniline and poly(3,4-ethylenedioxythiophene) nanofibres

PANI nanofibres were synthesized according to previous reports [27,28]. Briefly, N-phenyl-1,4-phenylenediamine (1.74 × 10⁻⁷ mol, Sigma-Aldrich, St Louis, MO, USA) in methanol was mixed with aniline (1.75 × 10⁻⁷ mol, Sigma-Aldrich) in 1 N HCl. Ammonium peroxydisulfate (4.39 × 10⁻⁴ mol, ThermoFisher Scientific, Waltham, MA, USA) in 1 M HCl was then added to the mixture. Polymerization proceeded overnight and was purified by dialysing with cellulose membrane dialysis tubing (MW = 12 000, Sigma-Aldrich). Dialysis was performed for more than 10 h with two fresh water changes.

Chemical synthesis of the PEDOT nanofibres was performed according to previous studies [29–31]. Briefly, 3,4-ethylenedioxythiophene (EDOT) monomer (7 × 10⁻³ mol, Sigma-Aldrich) was added into 50 ml of 1 M camphor-10-sulfonic acid (CAS, Sigma-Aldrich) containing 1 ml V₂O₅ sol–gel until the EDOT was completely dissolved. Ammonium persulfate (5 × 10⁻³ mol, (NH₄)₂S₂O₈ Fisher) in aqueous 1 M CSA was then added to initiate the polymerization. The solution immediately turned dark blue. After 5 h of reaction, the PEDOT nanofibre precipitate was cleaned with 1 N HCl to remove the vanadium compound, filtered and dried under vacuum at 80°C (Isotemp vacuum oven model 281A, ThermoFisher Scientific) for 12 h to yield approximately 600 mg of PEDOT nanofibres.

The conductive nanofibres and V₂O₅ templates for PEDOT growth were characterized using a Philips 400 120 kV transmission electron microscope (TEM, Hillsboro, OR, USA) with a high tilt goniometer stage on carbon coated formav 200 mesh copper grids (Ted Pella, Inc., Redding, CA, USA). The PANI and PEDOT nanofibres were chemically analysed using a Fourier transform infrared (FTIR) spectrometer (Spectrum 400, Perkin
Elmer, Waltham, MA, USA) equipped with universal attenuated total reflectance sampling accessory (Perkin Elmer). The PANI nanofibres were also observed using a scanning electron microscope (SEM, Nova NanoSEM 450, FEI, Hillsboro, OR, USA) at a 10 kV accelerating voltage.

2.2. PC-12 cell culture and differentiation

PC-12 rat pheochromocytoma cells (passage number = 4–9, ATCC, Manassas, VA, USA) were seeded in RPMI 1640 media supplemented with 10% horse serum and 5% fetal bovine serum (FBS) and 1% penicillin/streptomycin (P/S) in a humid incubator at 37°C and 5% CO2. For differentiation, a low-serum medium (1% horse serum) supplemented with 5% FBS and 1% P/S and 50 ng ml−1 NGF (Promega, Madison, WI, USA) was used. This medium was introduced after 24 h of initial seeding. Neurite outgrowth was observed under a Zeiss AxioImager M1 fluorescence microscope (Carl Zeiss MicroImaging, Thornwood, NY, USA) for phase contrast images after culture in the differentiation media for 7 days. All reagents for cell culture were purchased from Invitrogen unless stated otherwise.

2.3. Human skeletal muscle cell isolation and culture

Human skeletal muscle tissues were obtained from biopsies of calf muscles of male patients undergoing surgery (age 35–65) following approval by the Institutional Review Board of Wake Forest University Health Sciences. Briefly, muscle tissues were minced into small pieces and incubated with Dulbecco’s modified Eagle’s medium at 37°C. After 48 h of incubation, enzymatic digestion was performed with collagenase II (0.1% w/v, Worthington Biochemical, Lakewood, NJ, USA) and dispase (4 mg ml−1, Gibco, Invitrogen, Carlsbad, CA, USA) at 37°C under 5% CO2 for 1 h in an orbital shaker. The resultant cells were collected from the previous mixture. The cell pellet was resuspended in the medium (Dulbecco’s modified Eagle’s medium containing 10% horse serum, 1% P/S) and plated in a tissue culture dish. After 2 h incubation, the unattached cells in the supernatant were transferred to a new dish to remove the fibroblasts. This process was repeated twice. The floating cells were then plated on another dish and incubated for 7 days. Isolated hSMCs were grown in tissue culture dishes with skeletal muscle growth medium (SkGM-2, Lonza Group Ltd, Basel, Switzerland) containing the basal medium plus recombinant human epidermal growth factor, insulin, gentamicin, amphotericin, fetaun, dexamethasone and bovine serum albumin supplemented with 5% FBS until approximately 70% cellular confluence. The population of hSMCs was seeded in the collagen solution with passage number of 1–7 in this study.

2.4. Three-dimensional cell-seeded collagen scaffold protocol

Cells were detached from the plate by enzymatic treatment with 1× 0.05% trypsin and 0.2 g l−1 EDTA (HyClone, ThermoFisher Scientific) in an incubator for 10 min before collecting the cell pellet. Cells were suspended in the mixture solution of 10× Eagle’s minimum essential medium (Gibco) and 10× reconstitution buffer (1:1) in an ice bucket. The 10× reconstitution buffer was made from 2.2 g sodium bicarbonate and 4.8 g HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) in 100 ml H2O, sterilized and maintained at −20°C until use.

To increase the cell seeding density and distribution, cells were loaded in the three-dimensional collagen gels, instead of the traditional practice of seeding the cells on top of the scaffolds. The cell-laden protocol was developed in-house. Briefly, type I collagen from calf skin (C857, Elastin Products, Owensville, MO, USA) at a concentration of 2.16 mg ml−1 was dissolved in sterilized 0.1% v/v acetic acid in deionized water under magnetic stirring in an ice bucket for approximately 1 h. The sterilized conductive polymer, PANI or PEDOT, was added at a concentration of 0.5, 1.5 or 3 wt% to the dissolved collagen solution. A sterilized solution of 0.1 M and 1 M NaOH (ThermoFisher Scientific) was added to the mixed collagen solution to adjust the pH to 7.4 and begin inducing fibril assembly of the collagen just before adding the cell suspension. The neutralized gel solutions were mixed with the cell suspension to obtain a solution with a final concentration of 2 × 105 cells per ml. The solution of 0.5 ml was gelled for further analysis, resulting in 1 × 105 cells per scaffold. The final mixed solutions of collagen, conductive polymer and cells were transferred to 24-well non-tissue culture plates and incubated for 1 h at 37°C in humidified air with 5% CO2 to allow fibril formation to occur in the three-dimensional conductive gel scaffolds. Additional medium (1 ml) was added to each well and only a partial medium change (0.5 ml) was performed on each subsequent day.

2.5. Electrical conductivity measurement

Electrical conductivity was measured (n = 3) at an ambient temperature using a Series 2400 Source Meter (Keithley Instruments, Inc., Cleveland, OH, USA) with 4-point probes (Mueller Electric, Akron, OH, USA) placed on the conductive collagen solutions and gels containing PC-12 cells. The cell-laden conductive collagen solutions were dropped on four gold-coated electrodes on glass slides with approximately 20 µm thickness. Resistance was then measured with the 4-point probes and conductivity was calculated using the thin films equation [32]. For a very thin sample with 4-point probe measurement, where sample thickness (t) is less than probe spacing (s), and R = V/I, the electrical resistivity (ρ) for a thin sheet can be calculated by ρ = πr(b2/V1), where R is the measured electrical resistance, V is the measured voltage, I is the applied current and t is the sample thickness (approx. 20 µm in this study). The electrical conductivity (σ) of a very thin sheet is the inverse of the sheet resistivity or σ = ρ−1. Measurements were obtained in triplicate before and after the glass slides were incubated under physiological conditions for 1 h to form the collagen gels.

2.6. Live/dead and MTS assays

To evaluate cell viability after gel fabrication, cells in the conductive collagen scaffolds were stained with a live/dead reduced biobead viability/cytotoxicity kit (L-7013, Molecular Probes Invitrogen). Old medium was replaced with 1× Hank’s balanced salt solution (HBSS; same volume), SYTO10 and ethidium homodimer-2 (1:1) dye from the live/dead kit were mixed in 1 ml of 1× HBSS. The 1× HBSS covering the scaffolds was removed before replacing with the diluted dye mixture and incubated in the dark at room temperature for 15 min. The samples were then washed three times with 1× HBSS before observing under a Zeiss LSM510 laser scanning confocal microscope (Carl Zeiss MicroImaging).

A CellTiter 96 AQueous Non-Radioactive Cell Proliferation assay (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium in the presence of phenazine methosulfate (MTS), Promega) was used to determine cell density in the gels on days 1, 3 and 7 after the initial cell seeding. The gels were placed in new well plates and washed three times with 1× Dulbecco’s phosphate-buffered saline (PBS). The samples were incubated with 100 μl of 10% v/v MTS assay at 37°C for 2 h. The formazan product, which was bioreduced by MTS in living cells, was read at an absorbance of 490 nm using a spectrophotometer (SpectraMax M5, Molecular Devices, Sunnyvale, CA, USA). The cell proliferation assay was run in triplicate (n = 3) for both PC-12 cells and hSMCs.
2.7. Immunofluorescence staining
The PC-12 cell line is an established model for NGF-induced neurite formation [33]. PC-12 cells in the three-dimensional conductive collagen gels were cultured for 14 days and immunostained to confirm cell differentiation. These cells were further tested for neurite outgrowth without the addition of NGF to the differentiation media. Rabbit polyclonal antibody of microtubule-associated protein-2 (MAP2, 1:100 dilution, Cell Signaling Technology, Inc., Danvers, MA, USA) was used to detect a neuronal phosphoprotein that regulates the structure and stability of microtubules, neuronal morphogenesis, cytoskeleton dynamics and organelle trafficking in axons and dendrites [34]. The mouse monoclonal neuron-specific anti-β-tubulin III (neuronal; 1:1000 dilution, Sigma-Aldrich) was used to detect β-tubulin III protein in the microtubules of the neuronal cells.

The conductive collagen gels were fixed with 4% paraformaldehyde (Polysciences, Warrington, PA, USA) at room temperature for 20 min and gently washed three times with 1× PBS. Cell membranes were then permeabilized with 0.1% Triton X-100 (Sigma-Aldrich) in PBS for 3 min. After rinsing three times with PBS, non-specific binding sites were blocked using a protein blocker solution (Dako, Carpinteria, CA, USA) at room temperature for 30 min. The cell differentiation markers (MAP2 and β-tubulin III) were then added to the sample and incubated at 4°C overnight. After incubation, cells in the conductive collagen gels were washed three times with PBS and incubated with Alexa Fluor 488-conjugated goat anti-rabbit secondary antibody (1:400 dilution, Invitrogen) or Texas Red conjugated mouse anti-horse antibody (1:200 dilution, Vector Laboratories, Burlingame, CA, USA) for 40 min at room temperature. Cell nuclei were marked using 4’,6-diamidino-2-phenylindole-containing mounting media (Vector Laboratories, Burlingame, CA, USA). Samples were visualized using a Zeiss AxioImager M1 fluorescence microscope (Carl Zeiss Microimaging).

2.8. Western blotting
The MAP2 and β-tubulin III protein expression of PC-12 cells on conductive collagen gels was quantified using western blot analysis. The mouse monoclonal anti-β-actin antibody (Sigma-Aldrich) was used for loading control of the three-dimensional cell-laden gels. Total protein was determined using a commercially available BCA protein assay kit (Pierce, Rockford, IL, USA). For western blotting, the three-dimensional gel scaffolds were added to an equal volume of Laemmli sample buffer (Bio-Rad, Hercules, CA, USA) and 2.63% v/v β-mercaptoethanol (Sigma-Aldrich), and then heated to 95°C for 5 min. Proteins (10 μl of total protein per lane) were separated by sodium dodecylsulfate–polyacrylamide gel electrophoresis (SDS-PAGE) on 8% polyacrylamide gels at a voltage of 120 V for 1 h and 20 min, and then transferred onto nitrocellulose membranes. The membranes were incubated with anti-MAP2 (1:1000), anti-β-tubulin III (1:2000) or anti-β-actin (1:15 000) antibodies overnight at 4°C. The protein marker was BenchMark pre-stained protein ladder (Life Technologies, Carlsbad, CA, USA). Horseradish peroxidase-conjugated secondary antibodies (1:1000) were purchased from Cell Signalling Technology. Chemiluminescent reagents were used to visualize the protein signals using a Fujiﬁlm LAS-3000 imaging system (R&D Systems, Inc., Minneapolis, MN, USA). The Integrated Density function (ImageJ software, NIH) was used to quantify the expression of individual proteins after normalized by β-actin protein expression.

3. Results and discussion
In this investigation, we synthesized conductive polymer (PANI and PEDOT) nanofibres and incorporated them with the cell-containing collagen solution, which allowed us to generate conductive collagen gels in a unique fashion at physiological temperature and pH. Our work was inspired in part from the findings of Jun et al. [14], where they demonstrated myoblast differentiation on electrically conductive fibres without any electrical stimulation. Therefore, we investigated two cell types (PC-12 and hSMCs), which have been shown to respond to electrical cues. Specifically, we were interested in PC-12 differentiation only without any electrical stimulation as well as in the absence of NGF. Also, Abidian et al. [5] showed that they achieved axonal regeneration in vivo with a hybrid PEDOT–agarose hydrogel, which although requires deliberate attempts and is harder to prepare, does indeed possess a similarity in concept to our conductive gels. Thus, we also intended to improve on existing protocols for conductive hydrogels. The findings of this study are discussed in the following sections.

3.1. Polyaniline and poly(3,4-ethylenedioxythiophene) nanofibres
The TEM results indicated that the chemically synthesized PANI and PEDOT have a nanofibrous structure (figures 1a,b and 2a,b, respectively). The diameters of both PANI and PEDOT nanofibres were in the nanoscale, which was observed to be less than 100 nm. The PANI nanofibres were around 500–1000 nm in length and about 40–50 nm in width with a needle-like structure. An SEM image of PANI nanofibres is also shown in figure 1c. The PEDOT nanofibres were about 500 nm in length and wider than PANI nanofibres at 50–60 nm in diameter. These measurements are from the TEM images (no distribution measurements were done). Usually, PANI can be synthesized from an aniline monomer by chemical polymerization without any template. In this study, the PANI nanofibres were obtained by an oxidative polymerization of aniline using as oxidant ammonium peroxysulfate. It was reported that PANI nanofibres with a diameter of about 50–60 nm could be obtained when using sodium chlorite as an oxidant [35]. Another study reported that PANI nanofibres synthesized by using potassium biiodate and sodium hypochlorite oxidants had an average diameter of approximately 50 nm and length of approximately 4 μm with higher electrical conductivity than using ammonium peroxysulfate oxidant [36]. PANI can exist as salt or base in three isolable oxidation states. The emeraldine salt is electrically conductive while leucoemeraldine, the fully reduced state of polyaniline, and pernigraniline, the fully oxidized state, are insulators [37]. In this study, the synthesized PANI nanofibres using ammonium peroxysulfate oxidant were observed to be green in colour or emeraldine, the half-oxidized state. The PANI nanofibres can be chemically polymerized into nanofibres without templates; however, the PEDOT nanofibres require V2O5 nanofibres as their growth templates. The V2O5 nanofibres were also imaged and shown in figure 2c, which were much longer (few micrometres) and thinner (20–30 nm) than PEDOT nanofibres. Zhang et al. [29] found that with the presence of 1 M CSA, V2O5 nanofibres dissolve more slowly, and the EDOT monomer dissolves completely resulting in a homogeneous reaction mixture. In that study, thus, the bulk morphology of the electrically conductive PEDOT nanofibres was precipitated from granular to nanofibrillar with a few micrometres in length and 100–180 nm in diameter. This phenomenon was also observed in this study with the navy blue coloured solution of chemically synthesized PEDOT nanofibres. The physical characteristics of PANI and
PEDOT nanofibres were very similar to what other researchers have described.

The FTIR spectra of the PANI and PEDOT nanofibres confirmed their signature chemical compositions. The amines appear in the IR spectrum of aniline monomers at 1004 cm\(^{-1}\), representing an N–H bond (primary amine) and at 754 cm\(^{-1}\), representing an N–H wag (primary, secondary amines; figure 1d). Peaks at 1498 cm\(^{-1}\) represent a benzenoid ring stretch and those at 1274 cm\(^{-1}\) represent a C–N stretch. The peaks at 1556.56 and 1454.22 cm\(^{-1}\) represent quinoid (Q) and benzenoid ring stretches in the PANI chains. While the peaks at 1268 and 1233.24 cm\(^{-1}\) represent C–N and C–N\(^+\) stretches, the peaks at 992 and 734 cm\(^{-1}\) represent an N=Q=N vibration and C–H deformation (out of plane), respectively. These findings confirm the existence of PANI chemically synthesized from aniline monomers. For the PEDOT nanofibres, the peaks at 1594.75, 1468.78 and 1275.77 cm\(^{-1}\) represent stretching modes of C=C and C–C in 3,4-ethylenedioxythiophene.

Figure 1. (a,b) TEM imaging of chemically synthesized PANI nanofibres. Scale bars are 500 nm and 100 nm, respectively. (c) SEM imaging of PANI nanofibres. Scale bar is 1 \(\mu\)m. (d) FTIR spectra of aniline monomer and PANI nanofibres indicating the important chemical bonds.

Figure 2. (a,b) TEM imaging of chemically synthesized PEDOT nanofibres. Scale bars are 100 nm. (c) TEM imaging of chemically synthesized V\(_2\)O\(_5\) nanofibres (a growth template of PEDOT nanofibres). Scale bar is 100 nm. (d) FTIR spectra of EDOT monomer and PEDOT nanofibres showing the important chemical bonds.
the thiophene ring (figure 2d). While the stretching modes of the ethylenedioxy group were found at 1126.50 and 1073.68 cm\(^{-1}\), the deformation mode was observed at 914 cm\(^{-1}\). The peaks at 1003.68 and 714 cm\(^{-1}\) represent the vibration modes of C–S bond in the thiophene ring. The peak at 889.24 cm\(^{-1}\) appeared to be identical to those seen in vivo [38]. In vitro, collagen undergoes self-assembly into collagen fibrils (called fibrillogenesis) and cross-links into collagen fibril networks that are organized distinctively in different tissues [39]. The results from previous studies indicated that the initiation of self-assembly of type I collagen involved the non-helical end regions (or enzyme susceptible regions of collagen molecules) and removal of these ends with enzymes prolonged initiation [40]. In vivo type I collagen-containing fibrils do not form in the absence of fibronectin, fibronectin-binding and collagen-binding integrins, and type V collagen, and thus cellular mechanisms are in place to control what is otherwise a protein self-assembly process [41,42]. With the presence of cells, we hypothesize that the collagen matrix would be assembled and cross-linked with other necessary proteins in extracellular matrix in order to create biomimetic, hierarchical structure of tissues. It will be interesting to study how conductive polymer nanofibres could influence fibrils and network formation in collagen gels in vitro. Chemical cross-linking to improve architecture of collagen fibrils is another approach that has been used to improve the mechanical properties but slows down the bio-degradation rate of collagen gels [43,44]; however, it can be very useful for drug delivery applications [45,46]. The chemical reaction and by-products could have detrimental effects on cross-linked gel in term of biocompatibility [47]. Moreover, cells were shown to have a direct effect on fibril formation and cross-linking in vitro [48], which inspired us to fabricate the cell-laden three-dimensional conductive gel scaffolds in this study.

### 3.2. Conductive collagen gels

The conductivities of the cell-laden collagen solutions and gels are shown in figure 3. In the solution form, all solutions had measurable conductivities, but a contrast could be seen once they gelled. For example, the collagen solution (positive control, Col (+)) was highly conductive; however in the gelled form its conductivity was non-significant. This is expected owing to decreased ion migration, and thus a corresponding decrease in measured conductivity. By comparison, the samples that contained the electrically conductive polymers, PANI or PEDOT, their conductivities were similar between solution and gel samples for all respective concentrations. Thus, the incorporation of PANI and PEDOT nanofibres made the scaffolds significantly more conductive than the collagen positive control (figure 3).

![Figure 3](http://rsfs.royalsocietypublishing.org/)

**Figure 3.** (a) Electrical conductivity and (b) relative conductivity of conducting collagen scaffolds relative to collagen solution alone. Data represent mean ± s.e.m. Statistical analysis was calculated using one-way ANOVA. *p < 0.05 when compared with Col (+) solution sample; and *p < 0.05 when compared with Col (+) gel sample.

Although Schmidt et al. [2] and Abidian et al. [5] used electrically conductive scaffolds to increase neurite outgrowth, no conductivity measurements were investigated. Guarino et al. [6] measured the conductivities for PANI-poly(ethylene glycol) diacrylate hydrogels and found them to range from 7.6 \( \times 10^{-8} \) mS cm\(^{-1}\) for poly(ethylene glycol) diacrylate alone to 1.1 \( \times 10^{-3} \) mS cm\(^{-1}\) for 3 wt% PANI in poly(ethylene glycol) diacrylate. By comparison, our PANI-collagen hydrogels ranged from about 5 \( \times 10^{-2} \) mS cm\(^{-1}\) for 1 wt% to 10 \( \times 10^{-2} \) mS cm\(^{-1}\) for 3 wt%, and hence were much more conductive at least by an order of magnitude. Similarly, our PEDOT-collagen hydrogels ranged from about 4 \( \times 10^{-2} \) mS cm\(^{-1}\) for 1 wt% to 7 \( \times 10^{-2} \) mS cm\(^{-1}\) for 3 wt%. The results in figure 3 suggest that the electrical conductivity of the PANI-collagen gel was higher than the PEDOT-collagen gel for similar concentration. For example, at 3 wt% conducting polymer, the conductivity was about 10 mS m\(^{-1}\) for the PANI-collagen gel and about 7 mS m\(^{-1}\) for the PEDOT-collagen gel. The results showed that collagen gel is a barely electrically conductive material without conductive polymers. Therefore, an increase in conductivities occurs with higher weight percentage of the conductive polymers. It suggests that the conductivities in the gels are owing to PANI and PEDOT nanofibres alone.

Cells facilitate collagen fibrils to polymerize and cross-link with cell-secreted molecules and enzymes to form collagen fibre network. In vitro, the purified collagen solution spontaneously self-assembles (in gelling form) at room temperature in a buffer containing a neutral salt solution to form fibrils that appeared to be identical to those seen in vivo [38]. In vivo, collagen undergoes self-assembly into collagen fibrils (called fibrillogenesis) and cross-links into collagen fibril networks that are organized distinctively in different tissues [39]. The results from previous studies indicated that the initiation of self-assembly of type I collagen involved the non-helical end regions (or enzyme susceptible regions of collagen molecules) and removal of these ends with enzymes prolonged initiation [40]. In vivo type I collagen-containing fibrils do not form in the absence of fibronectin, fibronectin-binding and collagen-binding integrins, and type V collagen, and thus cellular mechanisms are in place to control what is otherwise a protein self-assembly process [41,42]. With the presence of cells, we hypothesize that the collagen matrix would be assembled and cross-linked with other necessary proteins in extracellular matrix in order to create biomimetic, hierarchical structure of tissues. It will be interesting to study how conductive polymer nanofibres could influence fibrils and network formation in collagen gels in vitro. Chemical cross-linking to improve architecture of collagen fibrils is another approach that has been used to improve the mechanical properties but slows down the bio-degradation rate of collagen gels [43,44]; however, it can be very useful for drug delivery applications [45,46]. The chemical reaction and by-products could have detrimental effects on cross-linked gel in term of biocompatibility [47]. Moreover, cells were shown to have a direct effect on fibril formation and cross-linking in vitro [48], which inspired us to fabricate the cell-laden three-dimensional conductive gel scaffolds in this study.

### 3.3. Cell viability

Cells in native tissue are surrounded by extracellular matrix and other cells, and hence stay in three-dimensional environment. This was our motivation for using a three-dimensional gel to mimic the natural organization in our investigation. Collagen...
gel can be used as a delivery system which transports stem cells and growth factors into a lesion for enhancing the tissue regeneration [49,50]. They are useful for the transplantation and differentiation of neural stem cells, and aligned arrangement of astrocytes \textit{in vitro} [51,52]. In this study, the three-dimensional conductive collagen gels were tested for their suitability as biomaterials to support cell proliferation and neurite outgrowth. The acid-soluble type I collagen solutions with conductive polymers were mixed with PC-12 cells or hSMCs at pH 7.4 as the cell-laden hydrogel solution before collagen fibrils were self-assembled and cross-linked at the physiological temperature and pH for 1 h. Cells demonstrated good viability in the three-dimensional conductive collagen gel scaffolds, suggesting that our gels successfully supported the growth of both cell types. Live cells were observed in all samples at day 7 of culture (figure 4). This indicates that scaffolds containing these conductive polymers were non-toxic. The confocal images were difficult to obtain owing to the presence of the conductive nanofibres, which are dark in colour, and hence obscure the visibility. However, we have included representative images in figure 4. Moreover, by adding PC-12 cells to collagen before the gelling process, cells were distributed homogeneously through the gels. The conductive collagen gels in this study showed potential for use as a cell carrier solution for cell delivery or three-dimensional cell-laden scaffold for recreating biomimetic tissue structure, which maintains the cell adhesive property of collagen. Further investigation of the effect of conductive polymer scaffolds was done through a quantitative analysis by using the MTS cell proliferation assay for both PC-12 cells and hSMCs.

### 3.4. Cell proliferation

On performing the MTS assay which analyses the cell viability and hence can be used to obtain a quantitative cell number, we found that cell proliferation was greater in conductive collagen gel scaffolds that contained lower concentrations of the conductive polymers, as clearly

![Figure 4](http://rsfs.royalsocietypublishing.org/Downloaded from http://rsfs.royalsocietypublishing.org/ on July 6, 2017)
observed at day 7 (figure 5a,b). PC-12 cell proliferation was significantly higher at day 7 compared with days 1 and 3 for all concentrations examined, indicating that neither PANI nor PEDOT nanofibres were cytotoxic at lower concentrations; however lower cell densities were observed when the conductive polymer concentration was increased (figure 5a). This finding suggests that a higher weight percentage of conductive polymers seems to have an inhibitory effect on cell proliferation. Cell proliferation in the Col-PANi 0.5 wt% scaffold was most similar in cytocompatibility to that of our positive control, Col (+), for PC-12 cells. For hSMC proliferation, cell densities at day 7 were significantly increased from day 1 for the collagen positive control, Col-0.5PANI, Col-0.5PEDOT and Col-1.5PEDOT (figure 5b). Furthermore, cell densities were comparable (no significant differences) between the collagen positive control, Col-0.5PANI and Col-0.5PEDOT at days 3 and 7 of culture. The cell density was slightly lower, however, in Col-0.5PEDOT than in Col (+) at day 7.

The electrical conductivity measurements revealed that conductivity was increased more than 400% in the gel form when 0.5 wt% of PANI or PEDOT nanofibres were added to the collagen. This suggests that Col-0.5PANI scaffolds could be approaching an optimal balance between imparting electrical conductivity while not inhibiting PC-12 cell and hSMC growth. Although all of the samples demonstrated a suitable environment for cells to grow and proliferate, the data suggest that a higher concentration of conductive polymer nanofibres may limit the growth potential for both cell types. Moreover, the PANI-collagen gels possess higher cell densities than PEDOT-collagen gels.

This study showed that cell-laden scaffolds could support cell growth and proliferation as an early step of biocompatible testing of the conductive collagen gel scaffolds. It can strengthen the confidence for further studies in cell differentiation of skeletal muscle and neuronal stem cells. Moreover, it will be interesting to investigate how cells in the scaffolds have changed the initial structure of the conductive type I collagen gels over time, and whether the mesh network of our scaffolds will be reorganized in the presence of laden cells. The results for the first time suggested that the conductive collagen gels were biocompatible with both cell types, which could be found in a neuromuscular junction tissue. A co-culturing study of skeletal muscle and motor neuron cells, which was used to model neuromuscular function [53,54], could also be done on these types of scaffold, because they have biomimetic structure inherited from collagen and attract cell adhesion via $\alpha_5\beta_1$ integrin cellular receptor, which contributes to collagen polymerization, for type I collagen fibrils [55]. They also possess comparable biocompatibility to collagen gel (control) when using low concentration (0.5 wt%) of PANI or PEDOT and facilitate electricity to pass through the conductive collagen gel.

### 3.5. PC-12 cell differentiation

PC-12 is a cell model with a reversible response to NGF, and thus they have been widely used to study neuronal differentiation in vitro. To observe the neurite outgrowth of PC-12 cells, NGF was added to low-protein media and cells were cultured on conducting collagen gels for 7 days. As expected, neurite extensions were observed in the Col-3.0PANI, Col-3.0PEDOT and collagen positive control with phase contrast microscopy after 7 days of culture (figure 6a–c). The result confirmed that NGF induces neurite outgrowth in these three-dimensional conductive collagen gels as well as in the control group. In the NGF-free media, neurite outgrowth of PC-12 cells was also observed in the three-dimensional conductive collagen gels, as shown in figure 6d–f. Therefore, further analysis of protein expression related to neurite outgrowth (dendrite and axon) of PC-12 cells was undertaken.

PC-12 cells sense and respond to substrates differently. Earlier studies demonstrated the influence of substrate factors, such as spatial, mechanical and chemical cues, on neural differentiation without NGF induction [56–59]. For example, Lamour et al. [60] found that on solid glass surfaces containing a nanoscale mixture of hydroxyl and amine groups, PC-12 cells had the ability to differentiate in NGF-free media. The roughness of nanostructured TiO$_2$ triggers neuritogenesis of PC-12 cells without NGF by activating
the expression of nitric oxide synthase and the phospho-
extracellular signal-regulated kinase 1/2 signalling [61]. It
was shown that a nanoscale chemical heterogeneity has a
critical influence on the differentiation of PC-12 cells on
rigid solid glass without NGF treatment [62]. The spatial dis-
tribution of surface potentials may also control neuritogenesis
as observed by PC-12 neurite outgrowth after only 24 h of
culture in the absence of NGF treatment [59]. Moreover,
there are other molecules that can mimic the action of NGF;
for example, fibroblast growth factors, which could promote
stable neurite outgrowth and neuronal differentiation in
culture of PC-12 cells [63]. Therefore, the presence of
nanoscale conductive fibres in our three-dimensional conduc-
tive collagen gels could play an important role in aiding
PC-12 cell differentiation in the NGF-free media in this study.

Upon NGF induction, PC-12 cells exhibited neurite out-
growth on the three-dimensional conductive collagen gels, as
expected. To examine whether any morphological changes
were induced by the conductive PANI and PEDOT nanofibres
in collagen gels without NGF induction, we investigated PC-12
differentiation by observing the neuronal-type structure
(dendrite and axon) with immunofluorescence staining. The
PC-12 cells were cultured for 7 days in low-serum media with-
out NGF. During the neurite outgrowth process, neurons
extend leading tips (growth cones), which sense the extracellu-
lar matrix topography and guide the directional structure of
the axons and dendrites [64,65]. Axons (extended long struc-
tures that transmit signals to the synapse) and dendrites
(branched structures around the cell body that receive signals
from other neurons at the synapse) are collectively called

**Figure 6.** Phase contrast imaging of neurite extension of PC-12 cells in the three-dimensional collagen gel scaffolds after 7 days of culture: (a) positive collagen control, (b) Col-3.0PANI and (c) Col-3.0PEDOT upon the addition of differentiation media. Phase contrast imaging of neurite outgrowth of PC-12 cells in the three-dimensional collagen gel scaffolds after 7 days of culture: (d) positive collagen control, (e) Col-3.0PANI and (f) Col-3.0PEDOT in NGF-free media. Arrows indicate observed neurites. Scale bars, 20 μm.

**Figure 7.** Immunofluorescence detection of PC-12 cells stained with anti-MAP2 (green) and anti-β-tubulin III (red) antibodies after 7 days of culture: (a) positive collagen control, (b) Col-0.5PANI, (c) Col-1.5PANI, (d) Col-3.0PANI, (e) Col-0.5PEDOT, (f) Col-1.5PEDOT and (g) Col-3.0PEDOT. Cell nuclei are stained with 4',6-diamidino-2-phenylindole (blue). Scale bars, 50 μm.
neurites in vitro. MAP2 is a dendrite marker and β-tubulin III is a neuron-specific axon marker which both can be detected in differentiated PC-12 cells [66,67]. The MAP2 and β-tubulin III were labelled green and red, respectively, as shown in figure 7. The observation of neurite outgrowth or cell differentiation was also confirmed by western blotting to measure relatively the MAP2 and β-tubulin III protein expression. The results suggested that PC-12 cells expressed MAP2 and β-tubulin III proteins more when cultured in the scaffolds containing PANI or PEDOT nanofibres than in the collagen scaffolds (positive control), as shown in figure 8a,b. MAP2 and β-tubulin III expression was detected at the least on the collagen positive control when compared with the conductive collagen samples (p < 0.05 by one-tailed t-test). There was no statistically significant difference in the MAP2 expression of PC-12 cells when cultured in the three-dimensional conductive collagen gels (figure 8a). The β-tubulin III protein expression was observed to be significantly different only between the Col-1.5PEDOT and the collagen gels containing either 0.5 wt% or 1.5 wt% PANI nanofibres (figure 8b), suggesting that the use of PEDOT nanofibres in the three-dimensional collagen gels may aid in PC-12 cell differentiation better than the use of PANI nanofibres. The β-actin protein expression was used as an endogenous control for western blot analysis to ensure the total proteins from each scaffold were loaded equally in the SDS-PAGE. The bands of tested proteins from all samples (figure 8c) suggested that the total proteins of all samples were equally loaded but there were differences in the amount of MAP2 and β-tubulin III expression. It means that MAP2 and β-tubulin III expression of PC-12 increases when cultured in the conductive collagen gels by using PANI and PEDOT nanofibres without NGF induction. The tested protein markers were not detected in the collagen negative control, confirming that all protein expression was contributed by PC-12 cells. Importantly, for the first time, these results demonstrated the differentiation of PC-12 cells into neuronal-type structures (dendrite and axon) by increasing MAP2 and β-tubulin III protein expression when cultured in the three-dimensional Col-PANI and Col-PEDOT gels after 7 days without NGF in low-serum media under standard cell culture condition.

Therefore, the nanofibred structure and surface chemistry of PANI and PEDOT may play an important role in PC-12 cell differentiation in our study. Future work could be a study of how this affects PC-12 differentiation, and how the network of collagen fibrils and conductive nanofibres were formed in vitro by reconstituted with neuron-like cells at physiological temperature and pH over time. It was found that cells were important in in vivo fibrillogenesis, aggregation and cross-linking. Thus, to mimic the extracellular matrix and cellular arrangement in tissue, one way is to use cell-laden collagen gels, which allow cells to grow, proliferate and differentiate. Thus, such collagen gel scaffolds may provide a better platform to develop functional tissue. The three-dimensional conductive collagen gels may also offer another system to study of the network formation of collagen in vitro with cellular aid.

Another achievement of our work is that for the first time a simple-to-use conductive hydrogel protocol can pave a way forward to further studies of conductive hydrogels for neural and muscular regeneration with ease and efficacy, as our results suggest. It is the first instance, to our knowledge, where differentiation of neuron-like cells (PC-12) has been shown without any electrical stimulation and NGF induction for the investigated time of 7 days in the three-dimensional collagen gels by using PANI and PEDOT nanofibres (positive control) when compared with the conductive collagen samples, as shown in figure 8. MAP2, β-actin and β-tubulin III expression was detected the least on the collagen positive controls. (c) Western blotting comparison of MAP2, β-actin and β-tubulin III expression of PC-12 cells cultured for 7 days between in the three-dimensional conducting collagen gels and in the collagen controls (positive and negative controls).

4. Conclusion

Conductive PANI and PEDOT nanofibres were successfully chemically synthesized and reconstituted with collagen and living cells in vitro. The three-dimensional conductive collagen gels demonstrated good cytocompatibility (cell viability and proliferation) with PC-12 cells and hSMCs through 7 days of the research.
observation. Specifically, at a concentration of 0.5 wt% PANI nanofibres, cell proliferation on the nanofibre-containing scaffolds was comparable to that on collagen controls for both cell types and the electrical conductivity was significantly increased (more than 400%) compared with collagen controls. The three-dimensional conductive collagen gels supported neurite outgrowth of PC-12 cells after 7 days of culture, suggesting that the cells differentiated in NGF-free media without electrical stimulation. Importantly, the findings of this study suggested that the presence of PANI or PEDOT nanofibres in the three-dimensional collagen gel scaffolds increased neurite outgrowth of PC-12 cells when compared with the collagen positive controls. Furthermore, these scaffolds could be stimulated with electrical signals, which might result in even higher cell density and differentiation. The results suggest that our gels could be potentially useful as conductive, biomimetic scaffolds for nerve and skeletal muscle regeneration.

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