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Development of Gelatin and Graphene-Based Nerve Regeneration Conduits Using 3D Printing Strategies for Electrical Transdifferentiation of Mesenchymal Stem Cells

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Abstract

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Disciplines

Biomaterials | Chemical Engineering | Molecular and Cellular Neuroscience | Molecular, Cellular, and Tissue Engineering

Comments

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Development of Gelatin and Graphene-Based Nerve Regeneration Conduits Using 3D Printing Strategies for Electrical Transdifferentiation of Mesenchymal Stem Cells

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Abstract

In this study, gelatin and graphene-based nerve regeneration conduits/scaffolds possessing tailored 3D microstructures and mechanical properties were fabricated using 3D printing. The effect of 3D conduit microstructure and mechanical properties along with the applied electrical stimuli on mesenchymal stem cell (MSCs) behavior and transdifferentiation into Schwann cell (SC)-like phenotypes were investigated. The results indicated that the gelatin conduits/scaffolds had favorable 3D microstructural and mechanical properties for MSC attachment and growth. Immunocytochemistry results demonstrated that the application of electrical stimuli through the conductive graphene within the gelatin-based 3D microstructure had a profound effect on the differentiation of MSCs to SC-like phenotypes and their paracrine activity. 80% of the cells exhibited SC marker staining, and the cells showed significantly enhanced nerve growth factor (NGF) secretion. These results suggest that the electrical stimuli applied within the 3D gelatin matrix enables enhanced differentiation and paracrine activity compared to transdifferentiation procedures involving electrical stimuli applied on 2D substrates and chemical stimuli applied in 3D gelatin scaffolds, leading to promising nerve regeneration strategies.

Introduction

Large peripheral nerve injuries (PNIs) affect 13 to 23 out of every 100,000 people each year, leading to possible long-term disability, reduced quality of life, and heavy economic and social burdens.[1] Over 200,000 peripheral nerve repair procedures are performed annually, resulting in approximately $150 billion spent in annual health-care dollars in the United States.[2] Currently autologous nerve graft-based treatment strategies are considered the gold standard, yet suffer from a number of limitations including the requirement of multiple surgeries, biological complexity, donor site morbidity, and lack of graft tissue; and are not able to facilitate satisfactory recovery.[3, 4] Schwann cells (SCs) and implantable nerve conduit-based therapies are a promising alternative; however, donor site morbidity, lack of availability and slow *in vitro* growth of SCs limit the clinical translation and potential positive outcomes of this strategy.[5-9] Mesenchymal stem cells (MSCs), isolated and derived from various connective tissue sources, hold considerable potential for cell-based nerve regeneration therapies using autologous transplantation.[10, 11] MSCs can be transdifferentiated *in vitro* into SC-like phenotypes with enhanced paracrine activity and can be used for peripheral nerve regeneration without any ethical concerns.[12-20] However, the current difficulties in providing scalable differentiation protocols, designing a cell-laden implantable conduit with desired 3D microstructural/mechanical properties that mimic the complex extracellular matrix (ECM) microenvironment, as well as difficulties in precise control of the final fate of the implanted cell population, limit the *in vivo* application and clinical use of MSCs for peripheral nerve regeneration.[10, 20, 21] In addition, most of the currently applied *in vitro* transdifferentiation procedures rely on the use of expensive chemicals and growth factors without deep mechanistic understanding of regulated proteins and cellular pathways. Furthermore, these procedures are mostly tested on 2D substrates, which do not represent the natural 3D microenvironment of the cells.[13, 14, 16, 20, 22] Therefore, the current knowledge of mechanisms of

transdifferentiation of MSCs in a controlled 3D microenvironment with tunable properties is significantly limited and should be expanded with alternative approaches.

In our previous study, we have successfully transdifferentiated MSCs into SCs with enhanced paracrine activity in the 3D microstructure of gelatin-based conduits via chemical induction.[23] We have also recently demonstrated the transdifferentiation of MSCs into SCs via electrical stimuli on conductive graphene-based 2D substrates.[24] Based on this background, here we propose 3D bioprinted gelatin and graphene-based conductive, biodegradable and implantable conduits with desired microstructural/mechanical properties, mimicking the ECM microenvironment for *in vitro* and potential *in vivo* transdifferentiation of MSCs into SCs and *in vivo* peripheral nerve regeneration via electrical stimuli. We hypothesize that the synergistic effect of 3D microstructural/mechanical conduit properties combined with electrical stimuli will enhance transdifferentiation of MSCs into SCs. We rationalize that the tunable 3D gelatin conduit microstructure will provide the desired ECM microenvironment for cellular attachment, growth, differentiation and paracrine activity, while the graphene circuit embedded in the gelatin matrix via 3D printing will enable conductivity and *in vitro* and potential *in vivo* differentiation via electrical stimuli. The overall objective of this study is to overcome the current challenges of *in vitro* stem cell differentiation and enable potential *in vivo* applications, which will then open the way for an ultimate goal of creating effectively applicable clinical PNI repair strategies via the proposed conduit design.

Materials and Methods

Preparation of Gelatin Conduits with Graphene Rods

Gelatin conduits were prepared by combining molding and thermally induced phase separation (TIPS) techniques, as described in our previous work.[23] Briefly, the mold was first assembled by inserting a conductive graphene rod (3.0 mm in diameter) (Black Magic 3D) into a Teflon tube (5

mm in diameter). The dimensions of the conduits and the integrated graphene rods can be adjusted by changing the mold and graphene rod diameters. Gelatin (Type B gelatin from bovine skin, Sigma) solution was prepared by dissolving 0.5 g gelatin in 10 mL of deionized water at 55 °C. Then, the gelatin solution was injected into the mold. The molds with gelatin were kept at -20 °C for 5 h for phase separation and incubated in cold ethanol (−18 °C) overnight. After that, the graphene integrated gelatin was removed from the mold, incubated in nonsolvent, 1, 4-dioxane, for 2 days and freeze-dried overnight. The obtained gelatin-graphene conduits were cross-linked using 1-ethyl-3-(3-dimethyl-aminopropyl) carbodiimide HCl (EDC) and N-hydroxy-succinimide (NHS) chemistry.[23]

Preparation of 3D Printed Graphene Circuits Integrated Gelatin Scaffolds

A conductive graphene PLA filament (Black Magic 3D) was used to 3D print the interdigitated circuit with desired dimensions. The CAD model of the designed interdigitated circuit was configured in Slic3r software to adjust printing parameters and continuous 3D deposition of computer-designed circuit was performed using a filament-based 3D printer (LulzBot TAZ 6). After fabrication, the graphene-based interdigitated circuit was embedded in a gelatin solution that had been prepared as described above. Then, the same scaffold formation and cross-linking steps were followed as described above.

Characterization of Conduits/Scaffolds

The 3D microstructure (pore size and % porosity) of conduits/scaffolds were observed via scanning electron microscopy (SEM, make and model of SEM?). The mechanical properties of the conduits/scaffolds were detected via dynamic mechanical analyzer (DMA). The swelling ratio was determined by measuring the weight of the conduits/scaffolds before and after dipping them in deionized water. The conductivity of integrated circuits was tested by building up a circuit to light a bulb and measuring the resistance.

Cell Culture of MSCs

Previously isolated Brown Norway rat MSCs[25, 26] were grown in minimum essential medium (α-MEM, Gibco BRL) supplemented with 20% fetal bovine serum (FBS; Atlanta Biologicals), 4 mM l-glutamine (Gibco), and antibiotic-antimycotic (Invitrogen)(this growth medium is referred to as maintenance medium, MM) and incubated at 37 °C and 5% CO_2 environment. The cells were sub-cultured when they reached 80% confluence, every 2–3 days.

Cellular Attachment and Growth

The MSCs were seeded ($5x10^5$ cells) to the conduits/scaffolds to evaluate their cellular attachment and growth within the conduit matrix. The cells were injected as a suspension via micropipet within the conduit matrix and incubated for 15 days at 37 °C and 5% CO_2 environment by changing the cell culture media every 2 days. During the incubation, cell viability, proliferation and growth was observed. After the incubation, the cells were fixed in 4% paraformaldehyde and labelled with Rhodamine Phalloidin and DAPI to detect the cellular morphology, proliferation, and interconnection within the 3D conduits.

Transdifferentiation

Rat MSCs were transdifferentiated *in vitro* into SC-like phenotypes using electrical stimuli, by following the previously mentioned protocols. [24] MSCs ($5x10^5$ cells) seeded in either gelatin conduits integrated with graphene rods, or the gelatin scaffolds with graphene-based interdigitated circuits embedded within. Then, the cells were grown within the conduit microstructures for 1-2 days. Following this, an electrical potential of 100 mV at 50 Hz was applied for 2 min per day for 10 days through the rod, while the same electrical potential was applied to the interdigitated circuit for 10 min per day for 10 days to transdifferentiate MSCs into SCs. These

electrical parameters were selected based on our previous studies and the electrical signals and fields occurring endogenously during embryonic development.[20] MSCs ($5x10^5$ cells) seeded in the conduits/scaffolds and incubated in MM for 10 days without electrical transdifferentiation were used as controls.

Immunocytochemistry

The degree of transdifferentiation was evaluated by immunocytochemistry analysis using glial cell markers, calcium binding protein Rabbit-α-S100, Mouse-α-S100β (Abcam-ab11178), and low-affinity neurotrophin receptor Rabbit-α-p75 (Promega-G3231). The control and transdifferentiated MSCs within the conduit/scaffold microstructures were fixed in 4% paraformaldehyde. After washing with phosphate buffered saline (PBS), the cells were incubated with blocking buffer (5% normal donkey serum (NDS, Jackson ImmunoResearch, West Grove, PA, USA), 0.4% bovine serum albumin (BSA; Sigma) and 0.2% Triton X-100 (Fisher Scientific) in PBS) for one hour. Then, the primary antibodies of Rabbit-α-S100, Mouse-α-S100β and Rabbit-α-p75 (1:500) were applied to the samples and incubated at 4 °C overnight. The next day, the samples were washed with PBS and labelled with corresponding secondary antibodies, Donkey-α-Mouse-Cy3 (1:500, Jackson ImmunoResearch), Donkey-α-Rabbit-Cy3 (1:500, Jackson ImmunoResearch), and DAPI (4′,6-diamidino-2-phenylindole) (1:2000, Invitrogen) (for nuclei staining) in blocking buffer. After 90 min of incubation in the dark at room temperature, the samples were rinsed with PBS and fluorescence microscopy imaging was performed.

ELISA Test

The paracrine activity of the MSCs during the transdifferentiation process was also evaluated using specific ELISA test kits. Since SCs are known to secrete high amounts of nerve growth factor (NGF) upon injury, we primarily analyzed NGF secretion. During the transdifferentiation, cell culture media was collected every 2 days and the amount of NGF secreted was detected

through the corresponding ELISA kit (Abcam Rat Beta NGF ELISA kit ab100757) by following the manufacturer's protocol.

Co-culture with PC12-TrkB Cells

A non-contact co-culture system was used to evaluate the biological activity of secreted factors produced during the MSC transdifferentiation process. MSC ($5x10^5$ cells) laden gelatin scaffolds with graphene-based interdigitated circuits were co-cultured with PC12-TrkB cells[27] (provided by Dr. Moses Chao, Helen L. and Martin S. Kimmel Center for Biology and Medicine at the Skirball Institute for Biomolecular Medicine, New York Univ.) using non-contact trans-well plates (Corning transwell inserts Product #3412). The scaffolds with MSCs were placed onto the 0.4 μm pore size cell inserts, which were then placed above the PC12-TrkB cells seeded into 6-well plates. The co-culture media was composed of 70% PC12 cell culture media (RPMI-1640 supplemented with 10% equine serum and 5% FBS) and 30% of MM. During the co-culture, an electrical stimulus (100 mV at 50 Hz, 10 min per day for 10 days) was applied only to the MSCs seeded in the scaffolds. At the end of this incubation, the extent of neurite outgrowth from the PC12-TrkB cells was visualized under fluorescence microscopy after ICC analysis using primary antibody Tuj1 and secondary Alexa Fluor 488 (1:1000, Molecular Probes-Life Technologies, Carlsbad, CA, USA).

Results and Discussion

Characterization of Fabricated Conduit/Scaffold

In this, study, we successfully developed conductive gelatin conduits with integrated graphene rods **(Figure 1 A)** and gelatin scaffolds with embedded 3D printed graphene-based interdigitated circuits **(Figure 1 B)**. The image of graphene interdigitated circuit embedded in gelatin after 3D printing can be seen in **Figure 1 B**. The graphene rods and interdigitated circuits were completely solid and intact, surrounded with the gelatin conduit/scaffold. The graphene rods and interdigitated circuits had a resistance of ~50 kΩ and were conductive enough to light a LED bulb

(Figure 1 A and B). The gelatin conduit and scaffolds surrounding the graphene rods and interdigitated circuits had similar microstructure as seen in SEM images presented in **Figure 1 A and B**. These microstructures had 90% porosity with the average pore size of ~135 μm. These structures also underwent swelling when they were in contact with water and the swelling ratio was calculated as 830. The obtained conduits microstructures showed similarity with our previous work[23] since we applied same fabrication method. Therefore, the integration of graphene-based rods or 3D graphene circuits did not affect the conduit/scaffold structure.

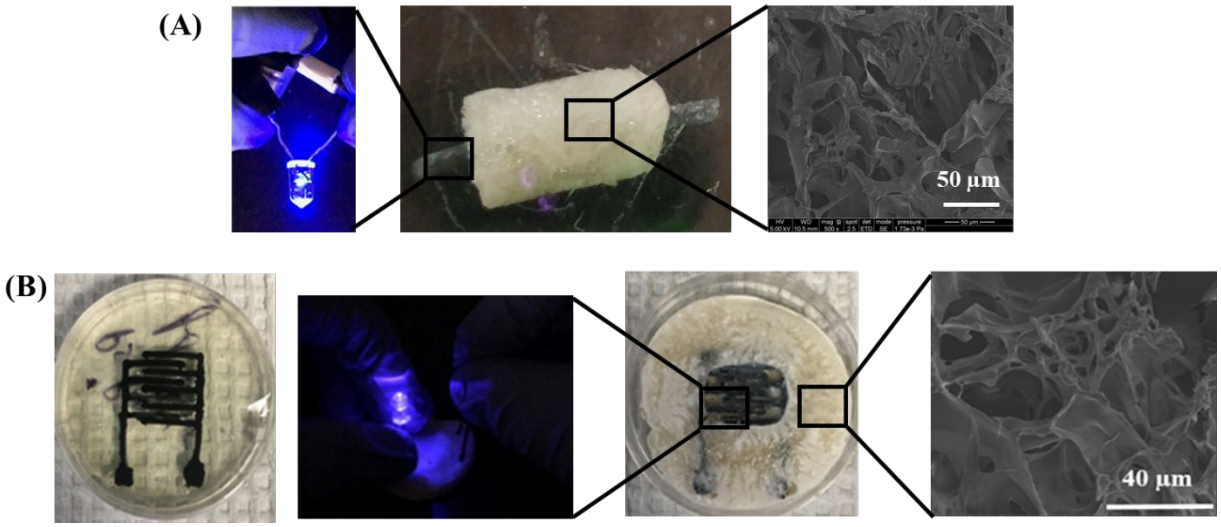


Figure 1. (A) Graphene rod integrated gelatin conduits. **(B)** Graphene-based interdigitated circuit embedded gelatin scaffold.

In addition to the microstructural properties, the mechanical features of the conduits/scaffolds were also evaluated. During this evaluation, the integrated solid graphene rods and circuits were not included and only the mechanical properties of gelatin-based structure was evaluated. The gelatin structure displayed an elastic behavior with storage modulus (G') higher than the loss modulus (G'') as shown in **Figure 2 A and B**. In addition, the G' and G'' values remained independent of the strain amplitude confirming the linear viscoelastic behavior **(Figure 2 A)**. The average complex modulus (G*, a measure of the stiffness) was calculated as 7.6 x 10^6 Pa (at a

frequency of 0.1 Hz, using 0.1% strain) while the loss factor (tan δ, a measure of the internal energy dissipation) was calculated as 0.11. Since G' value was higher than the G', the storage modulus (G') dominated the complex modulus (G*). These results indicated that the gelatin structure depicted an elastic behavior while exhibiting solid-like behavior at physiological temperatures (0 < tan δ 0.11 < 1). The typical stress-strain curve of the gelatin scaffolds was also measured **(Figure 2 C)**. The results indicated that the Young's modulus of the gelatin scaffolds was around 80 MPa with a yield strength of ~1.8 MPa, showing elastic behavior.

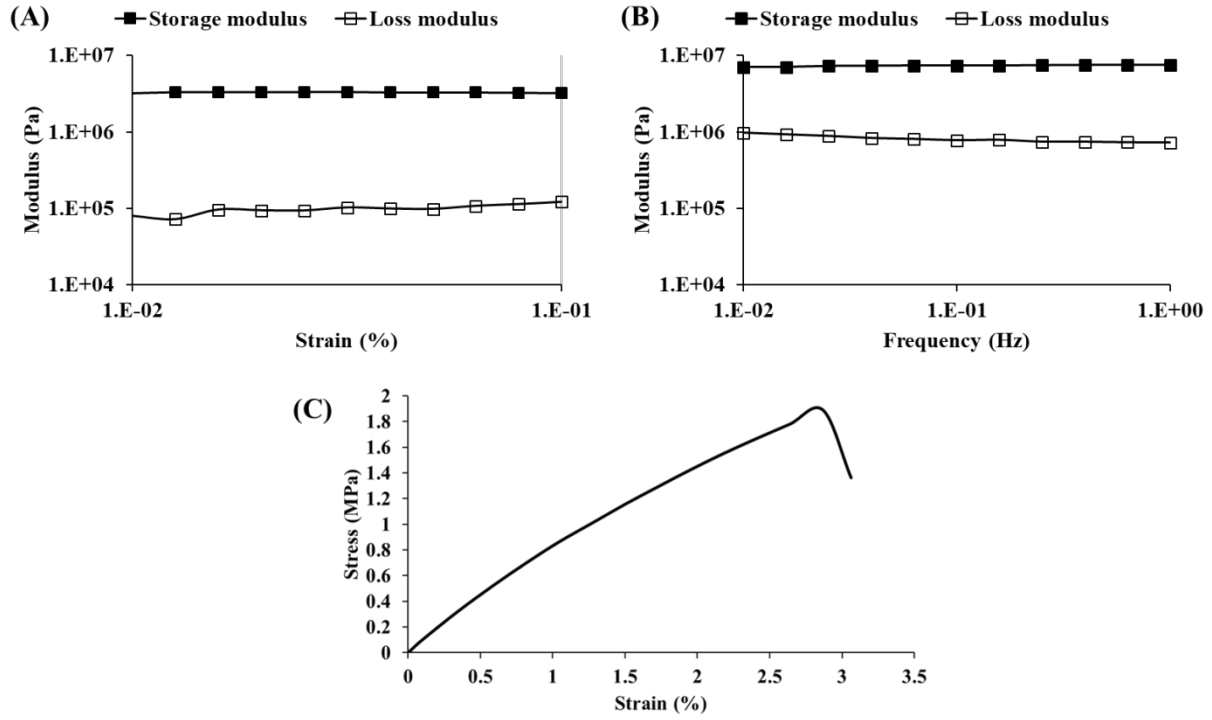


Figure 2. The storage (G') and loss (G'') moduli, as a function of **(A)** percent strain and **(B)** frequency. **(C)** Stress-strain curve of the gelatin scaffolds.

Cellular Growth and Differentiation of MSCs in the Conduit/Scaffold

The material-cell interactions were observed by seeding the MSCs in the gelatin structure. It was noted that the MSCs can attach, grow and proliferate within the 3D microstructure of gelatin matrix for more than 15 days without any issue of cell viability **(Figure 3)**. It was also observed that MSCs

created a 3D intercellular network within the porous 3D microstructure **(Figure 3 B)**. It is known that gelatin is a favorable material for cell attachment and growth. In addition, the 3D structure, pore size, porosity, mechanical elasticity and swelling property of the gelatin matrix has a profound effect on cell attachment and growth. The heterogeneous and anisotropic 3D structure can contribute to the recruitment of proteins to adhesion sites, which may also facilitate proliferation and spreading. [28-30] Matrix stiffness can also have an influence on the attachment, growth and differentiation along with the intrinsic mechanical properties of the cells as depicted in previous studies.[31-33] Together, these factors create a favorable microenvironment, mimicking the natural extracellular matrix, for MSCs.

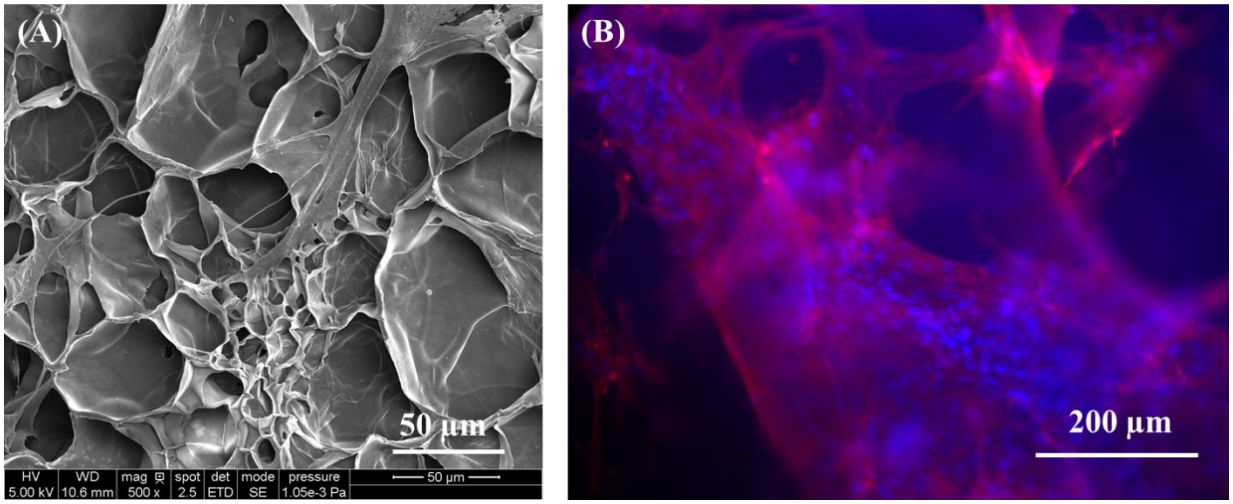
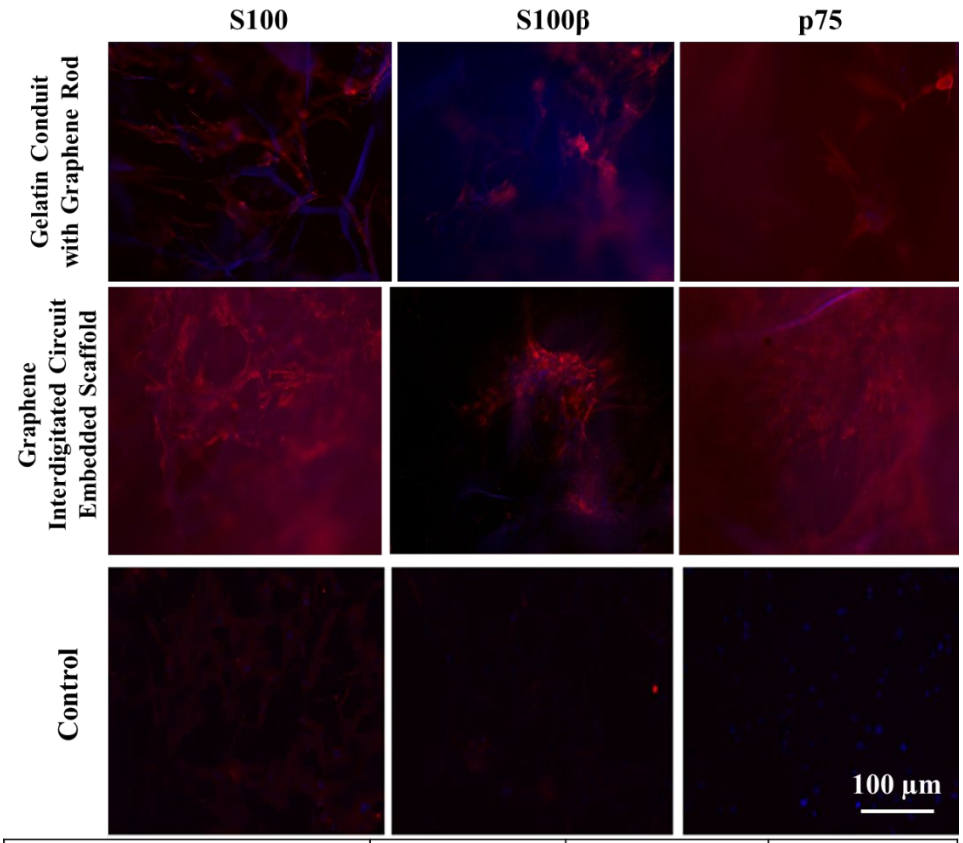


Figure 3. MSCs attachment, growth and proliferation within gelatin matrix. **(A)** SEM image. **(B)** Fluorescence image of Rhodamine phalloidin (red) and DAPI staining of MSCs within the gelatin scaffold. Scale bar in B, 200 µm.

The application of electrical stimuli within the favorable 3D gelatin microstructure resulted in enhanced differentiation of MSCs into SC-like phenotypes due to the synergistic effect of 3D microstructural/mechanical cues and electrical stimuli. We conducted an ICC analysis using s100, s100β and P75 antibodies as SC-markers considering the reports indicating their expression in

Schwann cells and regulation during injury and differentiation. [34] [35, 36] The ICC results indicated that MSCs seeded in the gelatin conduits with integrated graphene rods showed low degree of differentiation with 60% immunolabeling of S100, 38% immunolabeling of S100β and 18% immunolabeling of P75 SC-markers **(Figure 4, first row)**. This could be attributed to the electric field created in the gelatin matrix through the conductive graphene rod not being strong enough to support robust transdifferentiation. Considering this, we replicated the interdigitated circuit geometry from our previous work [24] on flat substrates, with different dimensions in the gelatin conduits using 3D printing. The ICC results showed that the MSCs, seeded in the gelatin scaffolds with 3D printed graphene interdigitated circuits, demonstrated significantly higher degrees of differentiation with 80% immunolabelling of S100, 84% immunolabeling of S100β and 75% immunolabeling of P75 SC-markers **(Figure 4, second row)**. This result is comparable to our previous studies where we transdifferentiated MSCs into SCs within a 3D gelatin structure using chemical induction, and obtained a degree of transdifferentiation of around 85%.[23] In another study from our group, we used 2D inkjet printed graphene interdigitated circuits on non-degradable polyimide substrates to transdifferentiate MSCs into SCs using electrical induction. The degree of transdifferentiation achieved with this approach was also around 85%. [24] Here, by combining the 3D microstructure and electrical stimuli, we were able to achieve around 80% of degree of transdifferentiation with electrical stimuli in a 3D microstructured conduit, which demonstrates the efficiency of our approach.

The transdifferentiation could potentially occur through promoting the upregulation of key neural gene expression patterns associated with focal adhesion kinase (FAK) signaling or mitogen-activated protein kinase signaling (p38) pathways [37-42] or via altering cellular membrane potential through hyperpolarization and depolarization, modification of ion channels, activation of calcium channels, and/or upregulation of the ERK pathway. [43-45] Other potential mechanisms might be through the activation of various signaling pathways such as MAPK, PI3K, and/or ROCK, [46, 47] and the increase in intracellular ROS generation. [46, 48]



	S100	S100β	P75
Gelatin Conduit with Graphene Rod	60.5%	38.3%	18.9%
Graphene Interdigitated Circuit Embedded Scaffold	80%	84.2%	75.7%
Control	26.6%	10.2%	0%

Figure 4. Transdifferentiation of MSCs in 3D conduits/scaffolds. MSCs in gelatin structure without any stimuli was used as control. Glial cell markers: s100, S100β and p75. Staining with Red: Cy3 and Blue: DAPI. Cell density: 5 x 10^5 cells per sample.

Another indication of successful transdifferentiation is enhanced paracrine activity. Schwann cells are known to secrete certain neurotrophic factors, particularly NGF, during injury that helps myelination and supports axonal regeneration.[14, 49] Considering this, we quantified the amount of secreted NGF during the transdifferentiation process. Our results indicated that the amount of NGF secreted from MSCs seeded in gelatin conduits with integrated graphene rods during

transdifferentiaion was around 0.175 pg/mL per cell **(Figure 5 A)**. This result was close to the control case and was expected, since the degree of differentiation obtained from ICC results for the MSCs in this conduit was also low. It is anticipated that this could be due to the insufficient electrical field created in the gelatin conduit using the conductive graphene rod. In contrast, the MSCs transdifferentiated within gelatin scaffolds with embedded 3D printed graphene interdigitated circuits, secreted NGF amounts of ~5 pg/mL per cell, which is significantly higher than the other counterpart and control groups **(Figure 5 A)**. In addition, this was also found to be considerably higher than the NGF secretion obtained in our previous studies, which varied between 0.5 to 2.5 pg/mL per cell. [23, 24]Therefore, the synergistic effect of 3D microstructural and mechanical properties and electrical stimuli did not cause significant measurable improvements in the degree of differentiation; however, it significantly enhanced the paracrine activity. To further demonstrate the biological activity of the secreted NGF, we conducted a non-contact co-culture experiment with PC12-TrkB cells. The results indicated a significant extension in the PC12-TrkB cells neurites, calculated as 5 μm per cell, obtained when the PC12-TrkB cells were in co-culture with MSCs seeded in gelatin scaffolds with embedded 3D printed graphene interdigitated circuits exposed to electrical stimuli **(Figure 5 B)**. The control cells displayed little to no neurite outgrowth.

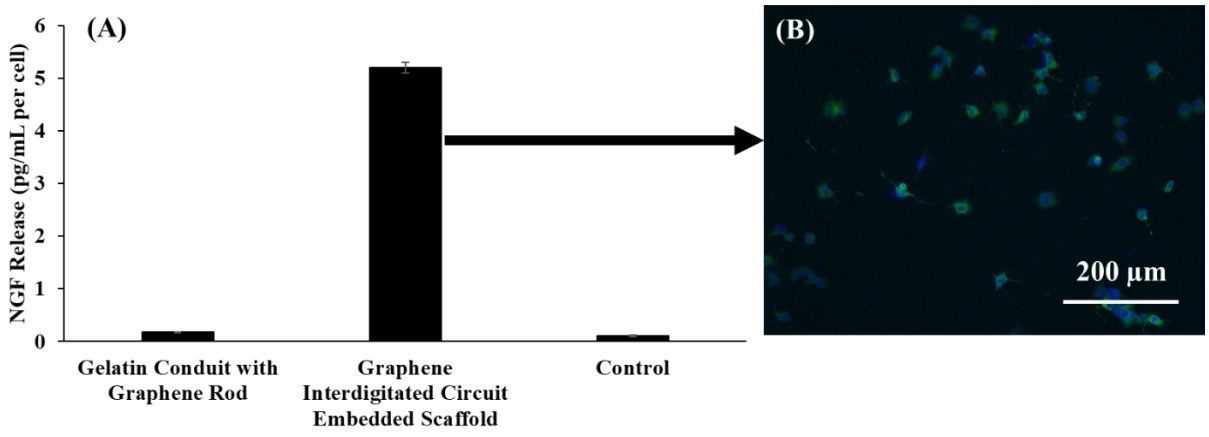


Figure 5. (A) Paracrine activity of MSCs differentiated in 3D conduits/scaffolds and secreted amounts of NGF. **(B)** Biological activity of secreted NGF on PC12-TrkB cells.

Conclusions

The synergistic use of 3D microstructural and mechanical cues in combination with electrical stimuli can be used as an alternative transdifferentiation strategy. This approach also eliminates the need for chemical stimuli-based conventional transdifferentiation protocols, which are non-scalable and potentially reversible, preventing direct *in vivo* application. Integration of 3D printed microcircuits within a scaffold structure can provide spatial and local control on cellular behavior. Moreover, this strategy can further be developed to enable potential in situ-*in vivo* control on the implanted cell population via electrical stimuli. The future direction will be to investigate different electrical stimuli parameters and obtain a clearer mechanistic understanding of electrical stimuli-based transdifferentiation. These results are promising and have potential to pave the way for alternative peripheral nerve regeneration strategies.

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