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Robust neurite extension following exogenous electrical stimulation within single walled carbon nanotube-composite hydrogels

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ABSTRACT

The use of exogenous electrical stimulation to promote nerve regeneration has achieved only limited success. Conditions impeding optimized outgrowth may arise from inadequate stimulus presentation due to differences in injury geometry or signal attenuation. Implantation of an electrically-conductive biomaterial may mitigate this attenuation and provide a more reproducible signal. In this study, a conductive nanofiller (single-walled carbon nanotubes [SWCNT]) was selected as one possible material to manipulate the bulk electrical properties of a collagen type I-10% Matrigel™ composite hydrogel. Neurite outgrowth within hydrogels (SWCNT or nanofiller-free controls) was characterized to determine if: (1) nanofillers influence neurite extension and (2) electrical stimulation of the nanofiller composite hydrogel enhances neurite outgrowth. Increased SWCNT loading (10–100-μg/mL) resulted in greater bulk conductivity (up to 1.7-fold) with no significant changes to elastic modulus. Neurite outgrowth increased 3.3-fold in 20-μg/mL SWCNT loaded biomaterials relative to the nanofiller-free control. Electrical stimulation promoted greater outgrowth (2.9-fold) within SWCNT-free control. The concurrent presentation of electrical stimulation and SWCNT-loaded biomaterials resulted in a 7.0-fold increase in outgrowth relative to the unstimulated, nanofiller-free controls. Local glia residing within the DRG likely contribute, in part, to the observed increases in outgrowth; but it is unknown which specific nanofiller properties influence neurite extension. Characterization of neuronal behavior in model systems, such as those described here, will aid the rational development of biomaterials as well as the appropriate delivery of electrical stimuli to support nerve repair.

Statement of Significance

Novel biomedical devices delivering electrical stimulation are being developed to mitigate symptoms of Parkinson's, treat drug-resistant depression, control movement or enhance verve regeneration. Carbon nanotubes and other novel materials are being explored for novel nano-neuro devices based on their unique properties. Neuronal growth on carbon nanotubes has been studied in 2D since the early 2000s demonstrating increased outgrowth, synapse formation and network activity. In this work, single-walled carbon nanotubes were selected as one possible electrically-conductive material, dispersed within a 3D hydrogel containing primary neurons; extending previous 2D work to 3D to evaluate outgrowth within nanomaterial composites with electrical stimulation. This is the first study to our knowledge that stimulates neurons in 3D composite nanomaterial-laden hydrogels. Examination of electrically conductive biomaterials may serve to promote regrowth following injury or in long term stimulation.

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1. Introduction

Since the 1800's, electrical stimulation has been applied to the nervous system as a potent stimulus to both induce movement and produce visual sensations of light with more recent biomedical applications directed toward stimulation of neural tissue to promote repair following injury [1]. During embryogenesis and following injury, cells are subjected to an endogenous voltage gradient generated by ion transport across the epithelium which impacts directed migration [2–6]. The manual disruption of natural current gradients during avian embryogenesis results in malformed limb buds, indicating the importance of this biophysical guidance cue for proper patterning and development [3,4,6]. Adult lower vertebrates, such as amphibians, have an innate regenerative capacity and following amputation in a newt forelimb, a transient gradient originating from the disrupted epithelium of 10–100 μA/cm^2 exits the regenerating stump tip [5,7,8]. Together, these results indicate endogenous currents are present following injury and if local cells retain responsiveness to these signals post-natally, they may be manipulated, influencing regeneration.

Neurons exhibit varying sensitivity to exogenous electrical stimuli *in vitro*. While some studies indicate no cellular response [9], other reported responses include: increased outgrowth [10,11], directional outgrowth (anode/cathode bias) [4,12–15], or increased branching [13]. Many of these studies utilized lower order model systems with known regenerative capacities (e.g. *Xenopus* limb regeneration), but limited knowledge of the mammalian neuronal response to electrical stimulation exists [16,17]. In recent work, we report an isotropic increase in neonatal rat sensory neurite outgrowth following exogenous stimulation with 50 mV/mm (1 mA, 8 h duration) relative to unstimulated controls [11,18]. The heterogeneity in reported responses to exogenous electrical stimulation make it difficult to predict outcomes, and may be due to varied sensitivity to stimulation parameters (e.g. duration, current) and/or inherent differences attributed to species, age, or cell type [19,20].

In vivo, oscillating electric fields have been used to treat canine spinal cord injuries and exhibited some promise in restoring ambulation. The beneficial effects, however, were not translated beyond human phase 1 clinical trials [21,22]. Application of alternating current (AC) electrical stimulation was recently shown to improve functionality via accelerated re-growth following post-nerve crush in carpal tunnel syndrome resulting in enhanced sensory and motor nerve regeneration in humans [23,24]. While promising, it remains unclear what factors are needed for translation to large-gap or more severe nerve injuries where natural structural and biochemical guidance cues are absent. Further, varying injury size and tissue composition likely impacts signal attenuation, providing the local cellular milieu with a variable electrical stimulus that may hinder translation from *in vitro* to *in vivo* platforms [21,22,25–27]. We hypothesize that the inclusion of an electrically-conductive biomaterial will mitigate signal attenuation and provide a more reproducible stimulus for a variety of wound geometries.

One possible method of manipulating bulk conductivity is to include an electrically conductive nanomaterial within the biomaterial. In this work, single-walled carbon nanotubes (SWCNT) were selected for their tailorable electrical, thermal, and mechanical properties to modulate conductivity within a model hydrogel [28–36]. The ability for carbon nanotubes to impact cell behavior in the absence of electrical stimulation has been of interest in recent years as the development of nano-bio interfaces gains traction in miniaturized medical applications. Both multi-walled carbon nanotubes (MWCNT) and SWCNT have been used for a variety of medical and clinical applications with promising results. However in the past these nanomaterials are generally used as coatings or within polymer composites for implants in applications such as bone regeneration, and with varying degrees of tissue response based on mode of application (inhalation vs. implantation or digestion) [37,38]. SWCNT are smaller in diameter (~1 nm), compared to the larger diameter MWCNT (4–65+ nm) and electrical and mechanical properties of each should be considered in detail prior to selection for the application, including chirality, mechanical strength, functionalization, biocompatibility, and conductivity [38–45].

In tissue engineering, nanomaterials have been used as both a substrate as well as incorporated in composite biomaterials. In a 2012 study, Martinelli and colleagues demonstrated an increase in cardiomyocyte viability, proliferation, and electrophysiological maturation when cultured on multi-walled carbon nanotube (MWCNTs) films [46]. Similarly, other electrically active cells, such as neurons, may also benefit from contact with nanomaterial interfaces. Stout and colleagues utilized electrospun poly(lactic-co-glycolic acid)(PLGA) carbon-nanofiber composites as a growth substrate for human cardiomyocyte and rat neuroblastoma cells, and found proliferation increased for both cell types [37]. In other work, both hippocampal and cortical neurons cultured on MWCNT films exhibited higher viability, growth, and synaptic output in the absence of applied electrical stimulation [47–49]. Hippocampal neurons increase postsynaptic currents and there is evidence of enhanced network efficacy, as well as increase action potential firing frequency on MWCNT films in comparison to both the nanomaterial free culture [50]. The inclusion of microscale features of conductive carbon nanotube ropes (1 mm diameter 1.5 cm length) with electrical stimulation elicited an enhanced neurite extension from neural stem cells on 2D surfaces [51]. In addition, tissue compliant MWCNT composites have shown benefit in neural interfacing applications, enabling acute recordings of neural activity [52]. Therefore, it is possible that electrically-conductive biomaterials may support or illicit more robust outgrowth or improved interfacing [53]. While the majority of the studies target cells grown on electrically conductive surfaces/biomaterials, the use of electrically-conductive injectable materials may be more readily translated for neural applications [54].

In previous work by Behan and DeWitt et al., nanomaterials, acid-treated single walled carbon nanotubes (SWCNT, 0.8–2 nm in diameter, 100–1000 μm in diameter; 0–50 μg/mL), were suspended throughout model 3D collagen-type I-35% Matrigel™ hydrogels and reported no change in primary Schwann cell viability or morphology over 2 weeks *in vitro* [55]. It remains unknown, however, if the nanomaterial will impact neurite outgrowth and/or if the measurable benefit of electrical stimulation to neurite outgrowth is lost [55]. In this work, neurite outgrowth from primary DRG was evaluated within a SWCNT-collagen type I:10% Matrigel™ composite hydrogels (0–100 μg/mL), in the presence or absence of electrical stimulation. Changes to neurite outgrowth were measured in response to both nanomaterial loading and electrical stimulation. Based on previous results demonstrating the benefits of electrically conductive materials with electrically excitable cells, we hypothesize that SWCNT-composite hydrogels will enhance neuronal outgrowth, and in combination exogenous with electrical stimulation we expect to see a further increase to neurite extension. To our knowledge this represents the first study to electrically stimulate primary neurons within a 3D, electrically conductive, nanomaterial-laden hydrogel.

2. Materials and methods

2.1. Isolation and culture of dorsal root ganglia

Whole dorsal root ganglia (DRG) were isolated from P2 neonatal rats as described previously (Sprague Dawley, P2; Taconic Farms,