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Aligned Microchannel Polymer-Nanotube Composites for Peripheral Nerve Regeneration: Small Molecule Drug Delivery

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Abstract

Peripheral nerve injury accounts for roughly 2.8% of all trauma patients with an annual cost of 7 billion USD in the U.S. alone. Current treatment options rely on surgical intervention with the use of an autograft, despite associated shortcomings. Engineered nerve guidance conduits, stem cell therapies, and transient electrical stimulation have reported to increase speeds of functional recovery. As an alternative to the conduction effects of electrical stimulation, we have designed and optimized a nerve guidance conduit with aligned microchannels for the sustained release of a small molecule drug that promotes nerve impulse conduction. A biodegradable chitosan structure reinforced with drug-loaded halloysite nanotubes (HNT) was formed into a foam-like conduit with interconnected, longitudinally-aligned pores with an average pore size of $59.3 \pm 14.2 \mu\text{m}$. The aligned composite with HNTs produced anisotropic mechanical behavior with a Young's modulus of $0.33 \pm 0.1 \text{ MPa}$, very similar to that of native peripheral nerve. This manuscript reports on the sustained delivery of 4-Aminopyridine (4AP, molecular weight 94.1146g/mol), a potassium-channel blocker as a growth factor alternative to enhance the rate of nerve regeneration. The conduit formulation released a total of $30 \pm 2\%$ of the encapsulated 4AP in the first 7 days. Human Schwann cells showed elevated expression of key proteins such as nerve growth factor, myelin protein zero, and brain derived neurotrophic factor in a 4AP dose dependent manner. Preliminary *in vivo* studies in a critical-sized sciatic nerve defect in Wistar rats confirmed conduit suturability and strength to withstand ambulatory forces over 4 weeks of their implantation. Histological evaluations suggest conduit biocompatibility and Schwann cell infiltration and organization within the conduit and lumen. These nerve guidance conduits and 4AP sustained delivery may serve as an attractive strategy for nerve repair and regeneration.

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Keywords

Peripheral nerve regeneration; small molecule drug delivery; sustained release; nerve guidance conduit; polymer composite; halloysite nanotube; sciatic nerve defect

1. INTRODUCTION

Peripheral nerve injury (PNI) often results in a loss of sensory, motor, and autonomic functions in the affected region. Moderate and severe PNI is often marred with very slow and frequently incomplete regeneration and can cause severe loss of sensory and motor function, where recovery is inversely proportional to the severity of damage [1–3]. Such injuries have a significant, and sometimes permanent, impact on patients and their day-to-day activities. Unlike cellular repair in other injuries, the wound healing response of the peripheral nerve does not involve mitosis and cellular proliferation [1]. In contrast, upon PNI, a calcium-mediated process known as Wallerian degeneration is initiated distal to the injury zone, starting with axonal breakdown, as a measure to alleviate abnormal axon regeneration [4]. Regeneration at the proximal stump begins within 24–48 hours, as a growth cone protrudes from the axonal stump and grows toward the target organ [5], but this endogenous repair usually does not sustain itself beyond 12 months [6].

Axonal regeneration is an extremely slow process that occurs at a rate of ~1mm/day and requiring at least 12–18 months for muscle reinnervation and initial functional recovery [7]. However, nerve gaps in the peripheral nervous system larger than 5mm often cannot regenerate naturally [8]. In the case of a critical-sized nerve defect, where the lesion extends too far away from the proximal end of the intact axon, regeneration to the distal stump is vitally slow and there is a great risk of traumatic neuroma formation, which not only renders the neuron ineffective, but is often very painful [9]. Those injuries which recover spontaneously (neurapraxic lesions) are thought to represent instances in which at least some of axons retain continuity through the lesion site [10]. If all axons are transected, then recovery depends on surgical intervention [11, 12]. Axonal and myelin sheath degradation stimulate proliferation and migration of Schwann cells and other neuronal support cells to the site of injury, forming conduits which guide regenerating axons to their target, known as the bands of Büngner [13, 14]. In addition to acting as a conduit, Schwann cells are a source of key neurotrophic factors, which exert trophic influence to which regenerating axons preferentially respond to, stimulating axonal regeneration by upregulating neuronal cell adhesion molecules and trophic cues [15, 16].

Current treatments for PNI depend largely upon surgical intervention, most popularly the use of autografts and allografts. These are limited by donor site morbidity and sensory loss, scarring, neuroma formation, limited supply, and potential immunosuppression [17]. Typical engineering approaches focus on synthetic or biological nerve conduits, however, due to variable, suboptimal outcomes these options are not often clinically utilized or used for noncritical small diameter sensory nerves only. Transient electrical stimulation has been extensively studied and has been reported to increase the speed of functional recovery after crush PNI due to increased nerve conduction. However, critical-sized nerve defects require

the use of nerve guidance conduits to bridge the gap and implementation of electrical stimulation poses a challenge in a clinical setting [18].

In an effort to mimic the effects of transient electrical stimulation, namely promotion of nerve impulse conduction via a pharmacological approach, the sustained release of a pharmacological agent that promotes impulse conduction may aid in regeneration of peripheral nerve following injury. A small-molecule drug, 4-Aminopyridine (4AP), with a molar mass of 94.11 g/mol is a voltage-gated potassium channel blocker and FDA approved in 2010 as AMPYRA® (Acorda Therapeutics, Inc.) for treatment of multiple sclerosis (MS). Due to its potassium channel-blocking abilities, it has been shown to prolong nerve action potentials and strongly promote neurotransmitter release [19–21]. Furthermore, direct administration has shown to enhance both speed and extent of functional recovery, as well as promote remyelination, following minor nerve crush injury [10]. However, the use of 4-aminopyridine has never been reported for applications in a segmental nerve defect, where there is a physical gap between proximal and distal ends of the nerve, or in any sustained-release formulations for such an application.

The ability of 4AP to block potassium channels causes delayed repolarization and allows it to prolong action potentials and amplify neurotransmitter release within neurons (Figure 1a). This serves to potentiate electrical performance and is the key factor which contributes to 4AP's clinical feasibility and utility as a therapeutic for MS [22], spinal cord injury [23], myasthenia gravis [24], and Lambert-Eaton syndrome [25]. The 4AP-induced blocking of potassium channels also has an effect on Schwann cells, which are present on the neurons and which migrate to the site of injury. The reduction of voltage-gated potassium channels has been correlated to the onset of myelin formation and developmental myelinogenesis [26, 27]. As such, a pharmacological reduction of potassium channels may have similar effects, leading to substantial myelin differentiation and neurotrophin release.

A number of natural and synthetic polymers are currently employed in biomedical applications, particularly as tissue engineering scaffolds and/or drug delivery systems. Most natural polymers are particularly attractive due to their inherent biocompatibility and biodegradability. Chitosan (Cht), the partially deacetylated derivative of chitin, is one such natural polymer derived from the shell of shrimp, crab, and other crustaceans. As such, it is abundant, affordable, and easy to commercially produce. Furthermore, several decades of polymer research have shown chitosan to be biodegradable, biocompatible, and possess antimicrobial properties [28–31]. Chitosan and its complexes have been studied for several biomedical applications including wound healing [32, 33], drug delivery [34], and implants [35]. These include applications related to nerve repair and regeneration [3, 36–38]. However, chitosan often lacks appropriate mechanical properties, particularly Young's modulus, for many biomedical applications. Its appeal as a hydrophilic polymer encourages the formation of hydrogels, however those limit the end-application, not having sufficient structural and mechanical integrity.

As such, we investigated the use of halloysite nanotubes (HNT) to form a stronger chitosan-HNT composite structure. HNT's have garnered major research attention over the last five years due to their nano-nature and intrinsic properties. Halloysite is a naturally-occurring

aluminosilicate nanotube and has been researched for applications as reinforcements in nanocomposites, where they serve to increase mechanical properties [39]. The neighboring alumina and silica layers, and their waters of hydration, curve and form multilayer tubes due to a packing disorder. They possess a hollow lumen (10–150 nm diameter) which provides excellent ability to carry, encapsulate, and transport chemical agents [39]. They can be effective in controlling the initial burst drug release typical of polymeric matrices and can prolong the sustained drug release, which remains a challenge for natural polymer systems.

In this study, we describe the fabrication, characterization, and testing of a porous chitosan sponge conduit scaffold, fortified with HNTs, with aligned micro-channel porosity as a drug delivery system and nerve guidance conduit (NGC) to bridge peripheral nerve defects, capable of guiding regenerated axons and delivering a sustained release of 4AP to the local target site.

2. MATERIALS AND METHODS

2.1 Materials

High molecular weight chitosan, halloysite nanotubes, epichlorohydrin, and FITC were purchased from Sigma-Aldrich (St. Louis, MO). Glacial acetic acid, sodium hydroxide pellets, phosphate buffered saline (PBS), LIVE/DEAD™ Viability/Cytotoxicity Kit, regenerated cellulose dialysis tubing with a MWCO of 3,500 Da, Nunc™ Lab-Tek™ II Chamber Slides, 4% Paraformaldehyde solution, Triton™ X-100, and NucBlue DAPI reagent were all purchased from Fisher Scientific (Fair Lawn, NJ). Normal goat serum, anti-NGF antibody (catalog #ab6199), anti-P0/MPZ antibody (catalog #ab61851), anti-BDNF antibody (catalog #ab108319), and goat anti-rabbit Texas Red (catalog #ab6719) were purchased from Abcam (Cambridge, MA). 4-Aminopyridine (>99%) was purchased from Alomone Labs (Jerusalem, Israel). Human Schwann cells and cell media were purchased from ScienCell Research Laboratories Inc. (Carlsbad, CA).

2.2 Halloysite nanotube drug loading

4AP-saturated solution was produced by dissolving 4AP in ultrapure distilled water at a concentration of 50 mg/mL at room temperature. Following dissolution, dry HNT was mixed with the 4AP-saturated solution in a weight ratio of 1:2. To encourage better dispersion and prevent precipitation, ultrasonication was employed for 1 hour. Cyclic vacuum pumping in/out was employed to enhance the replacement of air in the HNT's internal lumen with saturated liquid [40]. Vacuum was applied for 30 minutes, then the vacuum was broken, allowing 4AP solution to enter the lumen. This vacuum process was repeated three times and finally left overnight for higher drug loading. The HNT-4AP powder was separated by centrifugation (5000 rpm, 20 minutes) and washed with DI water three times to remove unloaded drug. Post-centrifugation, HNT-4AP powder was collected and freeze-dried for 24 hours to obtain dry powder. 10 mg of dry HNT-4AP powder was weighed and evaluated for drug loading using thermogravimetric analysis [39, 40].

2.3 Fabrication of composite nerve growth conduits

High molecular weight chitosan (Cht) (3.0% w/v) was dissolved in 2% (v/v) acetic acid solution at 50 °C in a water bath. The chitosan solution was stirred overnight until a homogenous solution was obtained. Air remaining in the solution is removed by vacuum pump for 24 hours. Conduits were prepared by injecting chitosan solution into custom-made molds of appropriate dimensions. The molds were subjected to 1 h of vacuum to remove air bubbles and allow equilibration at room temperature. The following unidirectional freezing was performed using a modified version of a published methodology [41]. The molds were then placed in insulating Styrofoam containers, such that only the bottom surface was exposed. The molds, covered with Styrofoam, were then placed onto the surface of a pre-cooled stainless-steel plate submersed in a 5 cm-deep pool of liquid nitrogen. As such, this created a uni-axial thermal gradient, which resulted in unidirectional freezing of the Chitosan solution (Figure 1). Samples were allowed to freeze for 30–45 min and were then subjected to freeze-drying to create to final structure. Random (unaligned) conduits were fabricated by simply freezing the chitosan solution overnight at –80 °C prior to freeze-drying. Similarly, samples with HNT and HNT-4AP were fabricated with the addition of 5.0% (w/w) of dry powder to the stirring chitosan solution. The freezing and lyophilization of HNT-4AP conduits was identical to that of neat chitosan conduits.

2.4 Crosslinking of Composite Conduits

Freeze-dried chitosan conduits were crosslinked using alkaline epichlorohydrin (ECH). Alkaline epichlorohydrin solution (0.01 mol/L ECH) was prepared in NaOH solution (0.067 mol/L) and samples were incubated in ECH solution at room temperature for 30 minutes [42, 43]. After this period, the samples were rinsed thoroughly with DI water three times to remove unreacted ECH. Samples were then vacuum dried to remove residual water.

2.5 Water Absorption (Swelling)

Crosslinked samples were tested for water absorption properties by soaking samples in 37 °C PBS (pH 7.4). Pre-incubation, samples were weighed to obtain initial dry weight. At predetermined timepoints, samples were removed from PBS, excess liquid was removed by gently tapping the samples onto Kimwipe paper and weighed to obtain the wet weight. The percent water absorption was calculated using the simple ratio of weights given in **Equation 1**, where W_t is the wet weight (g) and W_0 is the dry weight (g) prior to immersion in PBS [44].

$$\text{Water absorption (\%)} = \frac{W_t - W_0}{W_0} * 100$$

2.6 Conduit Porosity

Porosity of composite structures was calculated using a standard calculation of volume and mass, given by **Equation 2** [44], where V_m is total volume of samples (cm³), W_m is mass of samples (g), ρ is density of chitosan (1.342 g/cm³). For all porosity measurements, samples

(n=5) were formed in cylindrical molds and measurements of height and diameter were made in order to calculate total volume.

$$Porosity(\%) = \frac{V_m - \frac{W_m}{\rho}}{V_m} * 100$$

2.7 SEM Imaging

Surface morphology of samples was visualized and analyzed using scanning electron microscopy (SEM) (JEOL JSM-6335F, JEOL USA, Inc., MA, USA). Samples (n=3 for each formulation) were appropriately cut, adhered to carbon tape on SEM stub, and coated with Au/Pd using a Polaron E5100 sputtering system (Quorum Technologies, East Sussex, UK) for 3 minutes prior to imaging; This achieved a conductive layer of approximately 18 nm. ImageJ (NIH, Rockville, MD) was used to process and analyze images.

2.8 In Vitro Degradation Study

The degradation of conduit conduits (n=5) was studied in PBS (pH 7.4) and PBS+lysozyme at 37 °C. Conduits were initially weighed and immersed in PBS solution containing 4 mg/mL lysozyme at 37 °C for 12 weeks [45, 46]. Samples were collected after 1, 2, 4, 6, 8, and 12 weeks, washed three times in DI to remove ions adsorbed on the surface and freeze-dried. The freeze-dried samples were weighed and the degradation (mass loss) was calculated using **Equation 3** [47], where M_i is the initial weight (g) of the conduits prior to immersion and M_f is the final weight (g).

$$Mass\ loss(\%) = \frac{M_i - M_f}{M_i} * 100$$

2.9 Mechanical Testing

Crosslinked neat chitosan (0% HNT) and 5.0% HNT samples (n=6) were tested in the wet state by incubating in PBS (pH 7.4) overnight at room temperature prior to testing. Testing was performed on an Instron 3300 Single Column Universal Testing System (Instron, Norwood, MA, USA) at a tension rate of 5 mm/min. The testing yielded both tensile strength and Young's modulus results of the various samples.

2.10 Crystallinity (X-Ray Diffraction)

X-Ray diffraction (XRD) studies were performed using a Bruker D2 Phaser (Bruker AXS, Madison, WI, USA). XRD patterns for neat chitosan, neat 4AP, HNT-4AP, and composite CHT/HNT-4AP structures were recorded. Values were recorded from 5–40° (2θ) at a scanning speed of 0.2 deg/s. Cu-Kα (λ 1.546 Å) radiation at 40 kV and 30 mA was used as the X-Ray source.

2.11 Thermal Characterizations (DSC and TGA)

DSC thermograms were obtained for neat chitosan, neat 4AP, HNT-4AP, and composite CHT/HNT-4AP samples using a TA Instruments DSC Q100 (TA Instruments, New Castle, DE, USA). 10 mg of each sample in an aluminum pan was heated from 0–400 °C at a rate of 10 °C/min under 50 mL/min nitrogen purge. The DSC data was analyzed using the associated TA Instrument's Universal Analysis software package. Similarly, thermogravimetric analysis (TGA) of aforementioned samples was done using a TA Instruments TGA Q-500 (TA Instruments, New Castle, DE, USA). A 10 mg sample in a platinum pan was heated from 0–700 °C at a rate of 10 °C/min under nitrogen purge. Thermograms were recorded to capture weight loss as a function of increasing temperature.

2.12 Halloysite Nanotube Distribution, Alignment, and Imaging

In an effort to qualitatively determine the distribution of HNTs throughout the chitosan polymer matrix, HNTs were loaded with FITC in a similar manner as fully described above. Briefly, FITC was dissolved in acetone (1 mg/mL) and following dissolution, dry HNT was mixed with the FITC-saturated solution in a weight ratio of 1:2. The FITC-loaded HNTs were then centrifuged, freeze-dried to obtain powder, and later applied as 5% to the chitosan solution to create a composite structure. The structures were fabricated as described above, and thin circular slices were cut and the FITC-loaded HNTs were imaged using confocal microscopy. The images were processed and analyzed for directionality using ImageJ.

2.13 In Vitro Human Schwann Cell Viability

In order to assess the biocompatibility of chitosan conduits, an in vitro assessment was conducted using human Schwann cells at passage two. Cell viability was tested using a technique commonly known as live/dead staining, where kits combine fluorescent reagents to yield two-color discrimination of the population of live cells (green) from the dead-cell population (red). The LIVE/DEAD™ Viability/Cytotoxicity Kit was used and samples for live/dead assay were prepared by following assay manufacturer's instructions. Thereafter, confocal microscopy (Zeiss LSM 880, Carl Zeiss AG, Oberkochen, Germany) was performed for imaging.

Chitosan conduits were prepared as per the aforementioned method and sterilized under ultraviolet (UV) light for 1 hour on each side. Post-sterilization, conduits were soaked in appropriate medium for 1 hour prior to seeding 30,000 cells on top surface in untreated 24-well plates. Cell staining and imaging was performed 7 days post-seeding and confocal microscopy was used to obtain Z-stack images of conduits (10 slices, in 10 µm increments) in order to assess cell penetration within the 3D conduit and overall cell viability. Images were analyzed and maximum projection and gradient hyperstacks were created using ImageJ (NIH, Bethesda, MD).

2.14 In Vitro 4AP Drug Release

For 4AP drug release studies, the following groups were prepared as per aforementioned methods: (1) crosslinked composite: Cht+5% HNT-4AP, (2) composite: Cht+5% HNT-4AP, (3) crosslinked Cht+5% 4AP, (4) Cht+5% 4AP, and (5) HNT-4AP. All groups had a sample

size of $n=5$. Approximately 20 ± 2 mg of crosslinked and uncrosslinked samples were taken in regenerated cellulose dialysis tubing with a MWCO of 3,500 Da. Each bag was filled with 1 mL PBS, secured on both ends, immersed in 10 mL of PBS, and incubated in a 37 °C orbital shaker. For each formulation a sample size of $n=5$ was used. At various predetermined time intervals, 1 mL of the release media was collected and replaced with fresh media. The concentration of 4AP in the release media was determined by UV-Vis spectroscopy (Genesys 10S, Thermo Fisher Scientific, Waltham, MA, USA) at a λ_{max} of 260 nm using a standard curve previously created for 4AP in PBS [29, 48, 49].

2.15 Cell dose-response toxicity and proliferation

Human Schwann cells were used for all evaluations of cell dose response to 4AP treatments. Schwann cells, purchased frozen at passage one, were expanded to and harvested at passage two, as per the manufacturer's protocols. Cells were cultured on poly-L-lysine coated ($2\text{ }\mu\text{g}/\text{cm}^2$) tissue culture polystyrene (TCPS) well plates in manufacturer's Schwann cell culture medium comprised of basal media, supplemented with 5% fetal bovine serum (FBS), 1% penicillin-streptomycin solution, and 1% Schwann cell growth supplement. Cell cultures were maintained in a humidified incubator at 37°C and 5% CO₂.

For evaluation of potential toxicity effects, a dose-response study was performed with human Schwann cells using a lactate dehydrogenase (LDH) assay ($n=5$). The LDH assay is a strongly sensitive method for quantifying cytotoxicity, or degree of inhibition of cell growth, resulting from interaction of a test material with cells. Human Schwann cells were treated with five, varying doses of 4AP and tested for percent LDH leakage after 24 hours in culture [50, 51]. Dose-response studies evaluating Schwann cell proliferation were performed using a standard colorimetric MTS assay. A colored formazan product is produced by dehydrogenase enzymes in metabolically active cells. An MTS assay of human schwann cells was performed at day 1, 7, 14, and 21 with cells treated with 1, 5, and 10 $\mu\text{g}/\text{mL}$ of 4AP, compared to an untreated control which received no drug.

2.16 Immunofluorescent Staining and Quantification

For immunofluorescent staining experiments, human Schwann cells, harvested at passage two, were seeded onto poly-L-lysine coated ($2\text{ }\mu\text{g}/\text{cm}^2$) chamber slide wells at a concentration of 6,000 cells/ cm^2 . Cells were initially cultured using aforementioned Schwann cell culture medium, and media was replaced with 4AP-media solution (drug media) 24 hours post-seeding. Cells received drug media with 4AP in a concentration of 1, 5, or 10 $\mu\text{g}/\text{mL}$. These groups were compared to control groups where cells received standard Schwann cell culture media without drug. All cell media was changed every three days for the duration of the 14-day study ($n=3$).

At 14 days post-seeding, immunofluorescent staining was performed in order to examine the dose-response changes in Schwann cell proteins and neurotrophic factors, compared to control groups. Briefly, samples were washed using PBS (all washes performed in triplicate) to get rid of cell culture medium and were subsequently fixed for 30 minutes using 4% paraformaldehyde solution. Following fixation, samples were again washed in triplicate. Samples were permeabilized using 0.2% Triton X-100 prepared in PBS for 10 minutes then

washed with PBS. Samples were blocked by incubating with 10% normal goat serum in PBST (PBS with 0.1% Tween 20) for 30 minutes at room temperature followed by PBS wash. The primary antibodies, anti-NGF (1:200), anti-P0/MPZ (1:200), and anti-BDNF (1:1000) were all diluted to their respective ratios in the aforementioned blocking solution and added to each sample. Samples were incubated with primary antibody solution overnight at 4°C. The following day samples were washed three times with PBS followed by incubation for 1 hr with secondary antibody, Texas Red (goat anti-rabbit) diluted 1:2000 in blocking solution, and carefully protected from light. Samples were again washed with PBS and DAPI stain was applied prior to imaging using a Zeiss LSM 510 Meta confocal laser scanning microscope (Carl Zeiss, Oberkochen, Germany) [29, 52].

The resulting images were processed, analyzed, and quantified using ImageJ. To quantify relative NGF, P0, and BDNF fluorescence levels, a single in-focus plane was chosen for imaging. Using ImageJ, the area, integrated density, and mean gray value were measured for five independent images per sample, along with adjacent background readings. The total corrected cellular fluorescence (TCCF) = integrated density – (area of selected region × mean fluorescence of background readings), was calculated [53, 54]. This TCCF is presented as relative fluorescence of samples with arbitrary units, displayed as box-plots with 5–95% confidence intervals.

2.17 Animal Study Design

For a preliminary *in vivo* study to determine conduit surgical feasibility, suturability, and biocompatibility with regards to nerve regeneration, 12 female Wistar rats (Charles River Laboratories, Wilmington, MA) weighing roughly 200 g were randomly divided into two groups: sham and drug-conduit repair. All animals were housed individually with free access to food and drinking water in a temperature-controlled room with a 12-hour light-dark cycle. All animals were cared for and maintained according to methods approved by the Institutional Animal Care and Use Committee at the University of Connecticut and the National Institutes of Health regulations and standards for animal usage were followed.

2.18 Surgical Procedure

Each rat was anesthetized by inhalation of isoflurane and oxygen. The right hind leg was shaved and cleaned using betadine and isopropyl alcohol. A 30 mm incision was made parallel to the femoral axis and the right sciatic nerve was exposed through a gluteal muscle splitting incision. The sciatic nerve was carefully dissected free of surrounding tissues and a 15 mm nerve segment was sharply transected and removed. A 20 mm drug-loaded conduit was secured to the proximal and distal stumps using 8–0 Nylon monofilament suture (Ethicon Inc., Somerville, NJ), with approximately 2.5 mm of nerve stump inside the conduit. The muscle and skin incisions were closed with 5–0 Vicryl® suture (Ethicon Inc., Somerville, NJ). Pain was managed with injections of Buprenorphine every 12–18 h for two days post-operative. At 4 weeks post-operative, animals were euthanized by CO₂ asphyxiation. Sham-operated animals were treated identically as animals from the repair group, with the exception of leaving the sciatic nerve intact.

2.19 Histological Evaluation

Post-euthanasia, the right sciatic nerve was harvested and washed with PBS before being transferred to a histological container filled with 10% neutral buffered formalin and fixed at 4 °C overnight. Samples were then washed with PBS and transferred to 70% ethanol prior to embedding. Samples (n=3) were embedded in paraffin and cut to approximately 5 µm thick slices in preparation for histological assessments. Following standard protocols, hematoxylin and eosin (H&E) staining was performed on cross-sectioned and longitudinally-sectioned slices of native nerve and drug-loaded conduit repaired nerve and slides were imaged using an Aperio CS2 high-resolution digital slide scanner (Leica Biosystems Inc., Buffalo Grove, IL).

2.20 Statistical analysis

All data is expressed as mean ± standard deviation (mean±s.d.). All results were first evaluated using t-tests or one-way/two-way analysis of variation (ANOVA) followed by Dunnett's multiple comparisons test. All statistical analyses were performed with a confidence level of 95% ($p < 0.05$) using GraphPad Prism 7 (GraphPad Software, Inc. La Jolla, CA).

3. RESULTS

3.1 Conduit scaffold characterization

The aforementioned method of fabricating porous conduits resulted in highly porous, sponge-like scaffolds with specified geometries (Figure 2a). When imaged using SEM, a highly-aligned porous microstructure can be seen with microtubule-like pores aligned parallel to the longitudinal section (Figure 2b). Fenestrations can be seen interconnecting the longitudinally-aligned channels [55, 56]. As a result of the unidirectional freezing, and subsequent anisotropic growth of ice crystals, the cross-sectional images reveal elliptical forms, rather than symmetrical circles (Figure 2c). This is due to the fact that the cross-section of cellular ice crystals are not rotationally symmetric, but rather elliptical [55]. Given their elliptical geometry, measurements of pore Feret diameter, defined as the longest distance between any two points along the particle boundary, also known as the maximum size [57], yielded pores of 59.3 ± 14.2 µm (mean±s.d.). These findings are in agreement with previous studies which have investigated the effects of topography on axon regeneration and shown that pores sized 20–60 µm along the longitudinal direction were optimal for maximum axon penetration and minimum axon misdirection [56, 58].

These aligned and interconnected pores allow for longitudinal and horizontal migration of Schwann cells [56], which is critical for successful regeneration of nerve tissue, as well as a large surface area to provide local and sustained delivery of pharmacological agent to the site of injury [59]. The distribution of HNTs within the polymer matrix showed even distribution of aligned HNTs (Figure 2d). The directionality of HNTs was calculated using ImageJ, revealing HNT particles aligned preferentially to $90.6 \pm 16.6^\circ$ (mean±s.d.) with a goodness of fit R^2 of 0.99 (Figure 2e). The direction of alignment is rather arbitrary based on image orientation, however a strong goodness of fit to a particular direction is evidence of HNTs preferentially oriented in a particular direction, in this case the direction of polymer

alignment discussed previously. Similarly, when Schwann cells were cultured on conduits with aligned microchannel porosity, they show a clear favorable alignment correlating to the alignment of the polymer matrix (Figure 2f). The directionality calculations show that cells were aligned to $109.8 \pm 15.2^\circ$ (mean $\pm$ s.d.) with a goodness of fit R^2 of 0.97 (Figure 2g).

3.2 Mechanical properties

An investigation of the mechanical properties, namely Young's modulus (modulus of tensile elasticity) and tensile strength, of conduits with and without halloysite reinforcement was conducting using uniaxial tensile testing. Significant differences ($p < 0.05$) were found for both modulus and tensile strength with the addition of halloysite reinforcement. The Young's modulus was calculated to be 0.13 ± 0.02 and 0.23 ± 0.07 MPa (mean $\pm$ s.d.) for random (unaligned) conduits with 0% HNT (no halloysite) and 5% HNT, respectively. In contrast, the Young's modulus for aligned conduits were 0.18 ± 0.02 and 0.33 ± 0.09 MPa (mean $\pm$ s.d.) for aligned conduits with 0% HNT and 5% HNT, respectively (Figure 3).

3.3 Drug Loading Efficiency

Analysis of TGA profiles (Figure 4) indicated that 4AP degrades at $130\text{--}160^\circ\text{C}$ and the peak at 500°C indicates the presence of halloysite. At this temperature, halloysite does not fully degrade, but undergoes a loss of its hydroxyl group [40]. TGA profile of the halloysite drug composites, HNT-4AP (Figure 4b) shows the decomposition peaks for both 4AP and halloysite. The amount of 4AP in the composite was quantified as mass lost over the given temperature range and is expressed in percent of the total mass of the composite. TGA profiles constructed using the parameters identified from derivative curves indicated a 7.69 wt% overall drug loading. It has been commonly established in literature that successful drug loading of unmodified HNTs reached 5–10 wt% of the tube weight. Taking the density of the organic drug as 1.26 and halloysite 2.53 g/cm^3 , one could see that this value almost coincides with the complete filling of the lumen with condensed drug [39, 60].

3.4 In Vitro Degradation

The degradation of composite conduits was studied under physiologically-simulated conditions in PBS and PBS supplemented with lysozyme. The degradation rate of chitosan by lysozyme is inversely related to the molecular weight and degree of crystallinity of the chitosan, and proportionally related to its deacetylation [61]. Lysozyme recognizes and digests N-acetyl glucosamine sequences in the chitosan molecules, thus digestibility increases with increasing degree of N-acetylation in the polymer chain [62]. Following 12 weeks of incubation in PBS and PBS+lysozyme, $83.05 \pm 1.01\%$ and $70.99 \pm 1.27\%$ weight of samples remained (mean $\pm$ s.d.), respectively (Figure 5a). Inversely, this resulted in $16.95 \pm 1.01\%$ and $29.01 \pm 1.27\%$ weight loss, respectively. Weight loss in PBS+lysozyme at weeks 2, 4, 6, 8, and 12 were all statistically significant compared to that of in PBS. To supplement degradation data, samples subjected to PBS+lysozyme were collected at various timepoints and imaged using SEM (Figure 5b). Microscopy images show a consistent breakdown of the polymer microstructure, with fragmenting, cracking, and deterioration gradually increasing in relation the timepoint.

3.5 In Vitro Drug Release

Drug release from a variety of samples was tested in PBS (pH 7.4) at 37 °C. Loaded HNTs showed a sustained release of 4AP within 6 h (Figure 6b). When drug-loaded HNTs were incorporated into the chitosan polymer matrix (composite), sustained drug release was shown to be extended, with $94.68 \pm 3.62\%$ of drug being released within 168 h (7 days) (Figure 6c). This release was shown to be in stark contrast to that of the release of 4AP from chitosan polymer conduits without the incorporation of halloysite. In this case, where 4AP is simply mixed into the chitosan solution prior to fabrication, a $97.84 \pm 3.05\%$ release was shown within 0.5 h (mean $\pm$ s.d.). When this formulation, excluding halloysite, was crosslinked, a $58.19 \pm 2.79\%$ release was shown within 7 days. Lastly, formulations of crosslinked composite conduits, where 4AP-loaded HNT was incorporated into chitosan conduits and the resultant conduit was crosslinked, showed the longest sustained release of 4AP with $30.11 \pm 2.02\%$ release within 7 days. No significant differences in drug release properties were noted when drug-loaded chitosan conduits were exposed to UV during the process of sterilization. The structure of 4-AP released from conduits remained intact as evidenced through UV-spectroscopy and bioactivity.

Figure 6d illustrates the effect of the formulations on the early release of 4AP, focusing on the burst release, quantified as the percent cumulative drug release within the first 1 h. Drug-loaded halloysite (HNT-4AP) samples showed $88.20 \pm 6.62\%$, composite conduits showed $47.27 \pm 2.71\%$, crosslinked Cht/4AP conduits (no halloysite) showed $28.67 \pm 0.91\%$, and lastly crosslinked composite conduits showed the lowest burst release with $20.03 \pm 1.57\%$ drug release within the first 1 h (mean $\pm$ s.d.). Uncrosslinked samples of Cht/4AP which did not include halloysite were excluded from the burst release quantification as nearly 100% of drug was released in less than 1 h. Thus, according to the data representation, this would quantify as 100% burst release.

The drug release results from tested formulations were fit to various release kinetic models including Zero Order, First Order, Higuchi, and Korsmeyer-Peppas (K-P) (Table 1). In the K-P equation, the n value is the release exponent that indicates the mechanism of drug release. All three samples showed an $n < 0.5$, indicating quasi-Fickian diffusion of drug from the matrix [63], suggesting partial diffusion as the drug release kinetic. All four models show an interesting trend for the diffusion rate constant, k , where the Composite formulation showed the largest k value, crosslinked (CL) Cht/4AP showed the second largest, and CL Composite showed the lowest. Thus, calculations from all four models agree that 4AP release from a CL Composite Cht/4AP-HNT matrix was slightly slower than release from a CL Cht/4AP matrix, which was in turn, slightly slower than release from a Composite Cht/4AP-HNT. These results are also in agreement with the cumulative drug release plot discussed earlier in Figure 6c.

3.6 Human Schwann Cell Studies

In an effort to elucidate the effects of 4AP on physiologically-relevant cells, human Schwann cells were tested for dose-dependent responses to 4AP with regards to toxicity, proliferation, as well as expression of neurotrophic factors and proteins. The LDH toxicity assay, where the loss of intracellular LDH and its release (leakage) into the culture medium

is an indicator of irreversible cell death due to cell membrane damage [64], showed no statistical differences in LDH leakage between any of the 4AP treatment groups and the control group, which received no drug, indicating no cytotoxicity caused by the presence of varying doses of 4AP (Figure 7a). When analyzed for cell proliferation (Figure 7b), MTS assay of 4AP-treated Schwann cells cultured on TCPS for 1, 7, 14, and 21 days revealed conclusive dose-dependent inhibition, which is in agreement with previous literature [65].

MTS assay in conjunction with live/dead cell staining techniques were employed to study the viability, proliferation, and infiltration of Schwann cells cultured on 3D composite conduits. Over the course of 21 days, MTS assay of human Schwann cells seeded onto 3D composite conduits showed increases in cell number with significant proliferation by day 14 and day 21 (Figure 7c). Cell growth and proliferation over the 21-day period confirms the biocompatibility of the chitosan-based composite conduit system, as well as the composite polymer's ability to facilitate cell adhesion and proliferation. Given the 3D nature of the composite conduit, beyond cell adhesion and proliferation, cell infiltration into and within the 3D architecture was examined. At day 14, live/dead staining was employed to samples seeded with human Schwann cells. Z-stack images were taken at 10 μm slices and the composite images were projected and thresholded using a color-depth gradient (Figure 7d). Cell infiltration can be seen throughout the entire 100 μm projection with the majority of cells infiltrating 50–80 μm deep.

Schwann cell neurotrophic factor and protein expression was also investigated as a measure of dose-response to 4AP (Figure 8). Human Schwann cells cultured for 14 days with varying treatments of 4AP were subjected to immunofluorescent staining to assess the expression of three markers of interest: nerve growth factor (NGF), myelin protein zero (P0), and brain-derived neurotrophic factor (BDNF). Schwann cells were shown to exhibit dose-dependent increases in the expression of all three markers, compared to control groups, which received no drug. The dose-dependent increases in NGF, P0, and BDNF expression were shown qualitatively via immunofluorescent staining, and calculated as a quantification of immunofluorescent staining, expressed as relative fluorescence. NGF and P0 expression showed statistically-significant increases in both 5 and 10 $\mu\text{g/mL}$ 4AP-treatment groups, while BDNF expression increases were only significant in the 10 $\mu\text{g/mL}$ treatment group.

3.7 Preliminary In Vivo Studies

The primary focus of the preliminary *in vivo* rat sciatic nerve studies was mainly to establish the feasibility of suturability, surgical viability, and biocompatibility of the developed conduit constructs. At 4-weeks post-operative, all animals were sacrificed and conduits were examined and harvested for histological evaluation (Figure 9a/b). All conduits were observed to have remained in their surgical locations, attached to both proximal and distal nerve ends. There were no conduits found to have ripped from sutures and conduits showed minimal tearing or otherwise abnormal stresses, thus conduits have sufficient strength to withstand ambulatory forces.

The conduits were evaluated histologically using hematoxylin and eosin (H&E) stain using both cross-section and longitudinal sections of normal nerve from sham animals and nerve from those in drug-conduit group. Normal nerve shows typical wavy Schwann cell and axon

alignment and well-organized pattern surrounded by connective tissue Perineurium (Figure 9c/e). Regenerating nerve was found in drug-conduit groups, where there is early onset of infiltration and organization of Schwann cells through the conduit lumen and into the conduit matrix (where the conduit polymer is stained dark red) (Figure 9d/f).

4. DISCUSSION

The aforementioned fabrication method for polymeric nerve guidance conduit represents a straightforward method for achieving a tubular structure with aligned microchannel porosity for successful nerve regeneration. The use of the chitosan polymer not only grants natural biodegradability and biocompatibility, but also has a broad antimicrobial spectrum to which gram-negative, gram-positive bacteria, and fungi are highly susceptible [66]. In addition to its alignment, the composite system's incorporation of aluminosilicate halloysite nanotubes, which serve as an excellent drug-carrier given their hollow lumen, represents a highly effective method for the incorporation of small-molecule drug, in addition to mechanical benefits. The calculated HNT drug loading of 7.686% was well within the published range of 5–10 wt% of the tube weight [39, 60], confirming the complete filling of the tubes with condensed drug. Furthermore, silica-based scaffolds have been found to be effective for regeneration of soft tissues. Though the exact mechanism is unknown, it is believed that the silicate ions stimulate specific pathways responsible for the regeneration process [67].

A successful nerve guidance conduit should have a Young's modulus similar to that of native nerve tissues to provide sufficient strength and flexibility to be implanted and sutured during implantation, and to resist in vivo physiological loading and mechanical mismatch during nerve regeneration [68]. Although variations exist, it is generally accepted that Young's modulus of peripheral nerve, in the longitudinal direction, is in the range of 0.50 MPa [69]. In addition to its benefits as an excellent drug-release vehicle, the developed composite conduit's inclusion of halloysite nanotubes is an effective method to achieve tunable mechanical properties. Conduits with 5% HNT were shown to have a mean Young's modulus of 0.33 ± 0.09 MPa, very similar to that of native nerve. The increase in Young's modulus, with the increasing contents of HNT, are in agreement with other reports in literature [70, 71]. It is also important to note that no significant differences in tensile properties were noted when chitosan conduits were exposed to UV during the process of sterilization.

The composite, when processed via freeze-drying, resulted in a highly-porous, foam-like structure well-suited for many tissue engineering and regenerative medicine applications. Prior to freeze-drying, utilization of the unidirectional freezing technique resulted in consistent, longitudinally-aligned pores clearly visible in SEM images. The alignment of the chitosan polymer, and subsequently the halloysite nanotube fillers, resulted in mechanically anisotropic properties, preferential to the aligned longitudinal orientation. This is similar to the anisotropic structure of natural nerves, but extends also to muscles, tendons, blood vessels, and ligaments, which all have anisotropic mechanical properties [72, 73]. Furthermore, anisotropic pore structures have been correlated to excellent cell penetration, where highly aligned and porous scaffolds present significant contact guidance cues which promote cell motility within the network [74] – all of which are significant advantages with

regards to peripheral nerve regeneration. This was all evident in the seeding of human Schwann cells on the aligned microchannel conduits, where directionality testing confirmed Schwann cell alignment correlating to the polymer alignment.

Longitudinally-aligned pores within the conduit may serve as axonal tracts and provide directionality to the regenerating axon, in addition to providing increased surface area as compared to traditional tubular constructs. Given the hydrogel nature of the processed material, chemical crosslinking was necessary to stabilize the structure's integrity in aqueous environments. As an alternative to the most commonly used chitosan crosslinking agent, glutaraldehyde [75], which has been shown to be an irritant with known toxicity [76], epichlorohydrin was successfully used to crosslink the chitosan-based structure and stabilize its water absorption in aqueous conditions (Supplementary Figure 4).

According to the pathophysiology of peripheral nerve injury and repair, the first 7–21 days offer the greatest potential for facilitated regeneration as this is the period of Wallerian degeneration, cell recruitment, and the beginning of axonal regeneration. Release of 4AP using a hydrophilic polymeric nerve grafts is challenging. Many such scaffold formulations in our preliminary work released significant amount of 4AP (>75%) in the first 24 hours (data not shown). The primary purpose of this study was to solve this release problem along with scaffold suturability and its retention under animal leg movement at the defect site. 4-Aminopyridine release from fabricated conduits was shown to be dependent on the incorporation of halloysite nanotubes as well as the structure's crosslinking. Release of water-soluble drugs from natural polymeric matrices is often quick, while the release of small-molecule drugs is considered rapid; This was observed in samples excluding HNTs and crosslinking. 4AP-loaded HNTs showed a sustained release of drug within 6 h, in agreement with drug-release values from HNTs previously reported in literature [39, 60]. Crosslinking was shown to have a profound effect on sustained drug release, with the crosslinked composite structure containing chitosan and 4AP-loaded HNTs showed the slowest release. As such, the tunability of fabricated conduit system's drug-loading and drug release characteristics are highlighted, with optimized conduits releasing 4AP from well over seven days.

4AP treatment of human Schwann cells results in significant, dose-dependent upregulation of critical trophic factors, namely nerve growth factor (NGF), myelin protein zero (P0), and brain derived neurotrophic factor (BDNF), as shown by the immunofluorescent staining images and quantification of relative fluorescence. NGF is a neurotrophic factor expressed by migrating Schwann cells in developing or regenerating peripheral nerves and has been shown to stimulate the regeneration of axons along with BDNF [77, 78]. BDNF is a neurotrophin which has been shown to enhance peripheral nerve system myelination [79] and be required for peripheral nerve regeneration and remyelination after injury; studies show Schwann cells may contribute as the primary source of BDNF during regeneration [80]. Lastly, P0 is a major myelin protein critical in the development of myelin, which sheathes axons and acts as an electrical insulator, greatly speeding up action potential conduction. As such, all three factors are critically important to healthy regenerating nerves and their upregulation may be the key to more efficient and effective nerve regeneration. The MTS cell proliferation assay determined significant decreases in human Schwann cell

proliferation correlated to dose-dependent 4AP treatment. This 4AP-triggered decrease in proliferation corresponds to an increase in myelin differentiation and upregulation of neurotrophins and proteins, complimenting the aforementioned immunostaining data. These results are strongly in agreement with previous studies, which report that proliferation and myelin differentiation appear to represent two incompatible developmental possibilities, both *in vivo* and *in vitro* [81, 82].

In a preliminary *in vivo* study, where a 15 mm sciatic nerve defect was created in rats and bridged using drug-conduits, there were highly encouraging signs of nerve regeneration and axonal growth through the conduit lumen at 4-weeks post-operative. Histological evaluation using H&E staining showed initial growth and infiltration of cells through the conduit lumen, as well as infiltrating into the conduit matrix, with regenerating nerves showing similar patterns as native nerves collected from sham animals. The conduit was shown to have sufficient strength for suturability and to withstand all ambulatory forces, with no conduits tearing from surgical suture sites. Ongoing *in vivo* studies are characterizing the effect of the designed nerve guidance conduit in combination with 4AP in critical-sized 15-mm sciatic nerve defect in a rodent model over a period of 8 weeks. Nerve repair and regeneration will be benchmarked against appropriate controls including the nerve autograft (reversed nerve) and the neat conduit (no drug) in terms of quality of tissue healing (histology), myelin formation (microscopy), and functionality assessment *via* walking track analysis. We hypothesize that drug-eluting conduits with aligned microchannels will enhance the rate of nerve regeneration, superior to control conduit groups, and comparable to the autograft repair. In literature, many scaffold and conduit systems report on the incremental benefits of adding growth factors and cells to such systems in enhancing peripheral nerve regeneration [83–85]. Ongoing studies may result in a growth factor alternative conduit system applicable to nerve regeneration with superior clinical utility. Furthermore, the conduit system can be repurposed and optimized to deliver proteins, peptides, and other drug molecules to enhance the repair and regeneration of peripheral towards more efficient and efficacious functional recovery.

5. CONCLUSION

The developed polymer-nanotube composite drug delivery conduit demonstrates high potential for facilitating peripheral nerve regeneration. As an alternative to the conduction effects of electrical stimulation, we showed the design and optimization of a nerve guidance conduit with aligned microchannels for the sustained release of a small-molecule drug that promotes nerve impulse conduction. A critical challenge is the sustained release of small drug molecules using hydrophilic polymers, due to their faster diffusion rates. In this manuscript, we successfully demonstrate the sustained release of 4-Aminopyridine as a growth factor alternative to enhance the rate of nerve regeneration. The composite conduit released one third of the encapsulated 4AP in the first 7 days and the release was shown to be quasi-Fickian diffusion.

In vitro studies confirmed the beneficial effects of 4AP, increasing expression of key proteins such as nerve growth factor, myelin protein zero and brain derived neurotrophic factor in human Schwann cells. Preliminary *in vivo* studies in a critical-sized sciatic nerve

defect in Wistar rats confirmed conduit suturability and strength to withstand ambulatory forces over 4 weeks of their implantation. Histological evaluations suggest conduit biocompatibility and Schwann cell infiltration and organization within the conduit and lumen. Ongoing animal experiments look at qualitative repair and regeneration via histology and immunochemistry assays at throughout nerve healing under different treatment conditions and experimental controls. At these timepoints we are looking at functional recovery assessment via walking-track analysis. These nerve guidance conduits and 4AP sustained delivery may serve as an attractive strategy for nerve repair and regeneration.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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HIGHLIGHTS

- Chitosan nerve guidance conduit reinforced with drug-loaded halloysite nanotubes
- Longitudinally-aligned microchannels provide physical guidance and directional cues
- Sustained release of small-molecule drug 4-AP to enhance rate of nerve regeneration
- Schwann cells showed elevated expression of key neuroregenerative proteins
- May serve as a promising alternative to growth factors for nerve regeneration

Figure 1.

(a) Unidirectional freezing of chitosan solution was achieved by creating a uni-axial thermal gradient by exposing the bottom surface of chitosan solution in molds (insulated in Styrofoam, not shown) to a stainless-steel plate submersed in liquid nitrogen. Upon exposure, the uni-axial thermal gradient results in linear formation of ice crystals. (b) A simplified schematic illustrating the process of incorporating 4AP drug into Halloysite Nanotubes (HNT) and subsequently mixing with chitosan in a custom mold to produce drug-loaded conduits. A photograph of the prototype is shown along with representative SEM images of the aligned, porous microstructure.

Figure 2.

SEM images showing highly porous channel microstructure of (a) conduit cross sectioned, SB = 1 mm; (b) conduit sectioned longitudinally, SB = 100 μ m; (c) conduit cross sectioned, SB = 100 μ m. A highly linear pore configuration can be seen throughout the construct. (d) FITC-loaded HNTs were imaged within the chitosan matrix, showing an even distribution throughout the construct. (e) The directionality of HNTs was calculated, showing a highly aligned distribution of HNTs resulting from the alignment of the polymer matrix. (f) Human Schwann cells seeded on conduit showed alignment and proliferation in the direction of the

aligned polymer matrix. (g) The directionality of seeded Schwann cells confirmed an aligned Distribution of cells on the aligned microchannel conduit.

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Figure 3.

Tensile mechanical testing of fabricated nerve guidance conduits, both with aligned pores and random porosity, and with and without halloysite reinforcement (labelled 5% HNT and 0% HNT, respectively). Young's modulus is shown to increase in composite samples with 5% HNT as compared to samples with 0% HNT. Alignment of pores was shown to have anisotropic mechanical properties, increasing the modulus, particularly for the aligned composite with 5% HNT which showed the greatest moduli. Where native peripheral nerve is generally considered to have a Young's modulus of 0.50 MPa, aligned composite conduits showed very similar moduli. $\ast = p < 0.05$, $\ast\ast = p < 0.01$, and $\ast\ast\ast = p < 0.001$.

Figure 4.

TGA spectra (a) before and (b) after loading of 4AP into HNT. The modification of HNT loaded with 4AP (b) is shown as the TGA curve shows decomposition peaks for both 4AP and halloysite. The amount of 4AP loaded into HNT was quantified as mass lost over the given temperature range corresponding to the decomposition of 4AP and is expressed as the percent of total mass of HNT-4AP. (c) Pristine chitosan and composite Cht/HNT-4AP.

Figure 5.

(a) Degradation of crosslinked composite Cht/HNT-4AP samples in PBS pH 7.4 (black) and PBS with 4mg/mL lysozyme (red) (n=3 samples/timepoint, mean±s.d.). Degradation represented by the weight (%) of polymer remaining. *= $p < 0.05$ vs PBS, **= $p < 0.01$ vs PBS, and ***= $p < 0.001$ vs PBS. (b) Representative SEM images showing crosslinked composite samples at various timepoints during degradation in PBS+lysozyme.

Figure 6.

(a) Schematic of 4AP drug loading into HNT, followed by sustained release of drug from lumen. (b) Cumulative release of 4AP from halloysite nanotubes; (c) cumulative release of 4AP from Cht (black diamond), crosslinked Cht (blue square), composite Cht/HNT-4AP (red circle), and crosslinked composite Cht/HNT-4AP (orange triangle) (n=5 samples/group, mean±s.d.). (d) Burst release of 4AP from samples, where burst release is quantified as percent cumulative drug release within the first 1 h (n=5 samples/group, mean±s.d.).

=p<0.01, **=p<0.0001.

Figure 7.

(a) Lactate dehydrogenase (LDH) leakage cytotoxicity assay testing various doses of 4AP treatment on human Schwann cells in culture. ns=no significance. (b) MTS assay of human Schwann cells cultured on TCPS with no drug (control) or 1, 5, and 10 $\mu\text{g/mL}$ of 4AP for up to 21 days. $\ast = p < 0.05$ vs control, $\ast\ast\ast = p < 0.001$ vs control, and $\ast\ast\ast\ast = p < 0.0001$ vs control. (c) MTS assay of human Schwann cells cultured on composite conduits for 21 days. $\ast\ast = p < 0.01$ vs day 1. (d) Human Schwann cells cultured on composite conduits and stained using live/dead cell staining. A Z-stack projection of 10 confocal microscopy images, each 10 μm deep, is shown (i) and the same image is shown pseudo-colored for cell z-position using a color-depth gradient (ii).

Figure 8.

(a) In vitro immunofluorescent staining images showing human Schwann cells stained for NGF, P0, and BDNF (all stained red, respectively by row) in a dose response study to 4AP drug treatment. Control groups, which received media without drug, were compared to 1, 5, and 10 $\mu\text{g/mL}$ drug media solutions, respectively by column. SB=10 μm . (b) Quantification of immunofluorescent staining of NGF, P0, and BDNF (left to right, respectively). The intensity of staining obtained with each antibody was measured and displayed as box-plots

with 5 to 95% confidence intervals (n=5 images/group). $*=p<0.05$ vs control, $***=p<0.001$ vs control, and $****=p<0.0001$ vs control.

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Figure 9.

(a) Native rat sciatic nerve prior to transection. (b) 15 mm sciatic nerve defect surgically repaired with drug-loaded composite conduit. (c) H&E stain of a longitudinal section of native sciatic nerve shows well-organized myelin sheaths and round axons in wave formation, typical of axons and Schwann cells. (d) H&E stain of a longitudinal section of drug-loaded conduit repaired nerve 4-weeks post-operative. (e) H&E stain of a cross-section of native sciatic nerve shows well-organized axons and Schwann cells, surrounded by the connective tissue perineurium. (f) H&E stain of a cross-section of drug-loaded conduit

repaired nerve 4-weeks post-operative. Partial infiltration and organization of Schwann cells has begun through the conduit lumen and into the conduit scaffold matrix (stained dark red). The composite conduit scaffold shows robust suturability, sufficient ability to withstand ambulatory forces, and strong biocompatibility – with promising results that it is favorable to facilitate nerve repair and regeneration.

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Table 1.

Cumulative 4AP release data from respective samples fit to the Zero-order, First-order, Higuchi, and Korsmeyer-Peppas drug release models.

Sample	Zero-Order		First-Order		Higuchi		Korsmeyer-Peppas		
	k	R ²	k	R ²	k	R ²	k	R ²	n
Composite	0.263	0.983	0.00221	0.929	3.865	0.971	1.630	0.999	0.110
Crosslinked Composite	0.049	0.961	0.00013	0.964	0.725	0.962	1.278	0.918	0.080
Crosslinked CHT/4AP	0.161	0.984	0.00056	0.994	2.390	0.988	1.374	0.936	0.158