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Genipin-treated chitosan nanofibers as a novel scaffold for nerve guidance channel design

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ABSTRACT

Schwann cell-seeded nerve guidance channels are designed to assist post-traumatic nerve regeneration in the PNS. Chitosan is a natural polymer well suited for tissue engineering as it is biocompatible, non-immunogenic, and biodegradable. Electrospun chitosan nanofibers utilized in nerve guidance channels have the capacity for guiding axonal growth within the channel lumen yet are limited in their capacity to maintain structural integrity within physiological environments. To address this, we attempted genipin crosslinking of chitosan nanofibers. Compared to neat chitosan nanofibers, genipin-treated nanofibers exhibited increased stiffness, resistance to swelling and lysozymal degradation. Furthermore, alignment and proliferation of purified Schwann cell cultures upon genipin-treated substratum was enhanced. When dorsal root ganglion explants were utilized as an *in vitro* model of peripheral nerve regeneration, emigrating neurons and Schwann cells assumed the uniaxial pattern of aligned electrospun chitosan nanofibers. Neurite growth along the nanofibers led, reaching a frontier more than twice that of the pursuant Schwann cells. Critically, neurite growth rate upon genipin-treated nanofibers demonstrated a 100% increase. Altogether, genipin treatment improves upon the physical and biological properties of chitosan nanofibers towards their utility in nerve guidance channel design.

1. Introduction

Peripheral nerve injury sustained from trauma and iatrogenic injury can result in significant functional loss. The requirement for nerve sacrifice and reconstruction also occurs in the context of surgical oncology. Treatment of segmental nerve loss presents a treatment dilemma when primary repair cannot be achieved [1]. Autologous nerve grafting to bridge such defects is regarded as the 'gold standard' of treatment, but is limited by tissue availability, donor site morbidity, and size mismatch [2]. Nerve guidance channels (NGCs) have been designed as an alternative treatment option to bridge the nerve gap. Conduit design can be enhanced by the addition of internal topography to guide axonal regeneration [1,3]. Towards achieving this purpose, uniaxially aligned nanofibers have been generated from both synthetic and natural polymers via electrospinning. Another strategy towards enhancing conduit performance is to seed supporting cells types such as Schwann cells [4,5].

Chitosan (Cht) is a polymer of D-glucosamine and *N*-acetyl-D-glucosamine produced from deacetylation of chitin, a major component of crustacean shells [6]. It has emerged as an attractive candidate for tissue engineering because of its biocompatibility and biodegradability [6,7]. Generation of chitosan nanofibers via electrospinning requires dissolution in concentrated acids such as acetic acid or trifluoroacetic acid/dichloromethane [8], or alternatively by blending dilute acids with fiber-forming additives such as polyethylene glycol or poly(vinyl alcohol) [9,10]. Neat chitosan nanofibers however, are susceptible to degradation in physiological environments [7,11]. It is important that surface topography of the nanofiber scaffold is maintained as a guidance cue to growing nerves during the period of regeneration.

Treatment with genipin (GP) has been demonstrated to preserve the structural integrity of Cht nanofibers [12]. Genipin is a crosslinker derived from *Gardenia jasminoides Ellis* and demonstrates low cytotoxicity. It is unknown whether scaffold modification with GP is of benefit to neural regeneration. Here, we characterize the effect of GP treatment in enhancing nanofiber stiffness, resistance to swelling, and lysozymal breakdown. We demonstrate that Schwann cell cultures can be established upon a GP-treated chitosan nanofiber scaffold. In utilizing dorsal root ganglia (DRG) explants as an *in vitro* model of peripheral nerve

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regeneration, we show that uniaxially aligned GP-treated Cht nanofibers provide surface properties that speed up fiber-guided growth of axons (0.43 mm/day) well in advance of the follower Schwann cells (0.18 mm/day). At this rate, we expect 1-month for fiber-guided axonal regrowth to bridge a critical 12 mm gap in the sciatic nerve of a rat model and concurrently, about 2-months for Schwann cells to sort and remyelinate the regrowing axons. This contrasts the need for 3–4 months to achieve axonal regrowth and remyelination with a Schwann cell-seeded conduit in our previous study [4]. Given these properties, GP-treatment of Cht nanofibers improves upon scaffold design for a new generation of materials intended to be applied in nerve guidance channels.

2. Results

2.1. Fabrication of aligned chitosan nanofibers by electrospinning

Chitosan (Cht) nanofibers were fabricated under optimized dissolution and electrospinning parameters. To generate aligned fibers, coverslips were mounted onto a mandrel rotating at approximately 55 000 cm/min. In so doing, 80% of collected nanofibers achieved alignment within 20° from the main fiber axis (Fig. 1A and C). Fiber appearance and alignment remained similar subsequent to GP-treatment of the neat Cht nanofibers deposited upon coverslips (Fig. 1B).

2.2. Modification of chitosan nanofibers by genipin crosslinking

2.2.1. Fourier-transform infrared spectroscopy (FTIR)

Evidence of crosslinking between Cht and genipin (GP) was demonstrated by means of FTIR spectral analysis. Representative spectra of neat- and GP-treated Cht nanofibers were compared (Fig. 2A). In neat Cht nanofibers, absorption peaks at 1531 cm^{-1} and 1668 cm^{-1} are attributable to primary amine groups contained within Cht molecules [13–15], while peaks at 1089, 1141 and 1203 cm^{-1} are attributable to C—O and/or C—N stretching [14,15]. Given that crosslinking between GP and Cht involves the primary amine groups, the shift in peak to 1644 cm^{-1} with a corresponding decrease in absorbance was expected [12,13]. The broad peak at 1371 cm^{-1} reflects upon ring-stretching of the heterocyclic amine in the crosslinked Cht-GP product [16]. Another shift in peak from 1203 cm^{-1} to 1068 cm^{-1} can be attributed to C—N bond formation between GP and Cht, distorting the C—N stretch of neat Cht nanofibers [14,16].

2.2.2. Fiber appearance, diameter and stiffness

Macroscopically the GP-Cht nanofibers acquired a blue-green hue. Under flourescent microscopy, they emitted red autofluorescence that allowed for fibers identification in subsequent experiments (Fig. 6D). In contrast, neat Cht nanofibers were white and non-fluorescent. Based on SEM images, crosslinking resulted in a slight increase in fiber diameter from 215.3 ± 11.8 nm in neat Cht fibers to 281.7 ± 18.0 nm following treatment with 0.5% genipin (Fig. 2B).

Substrate stiffness is of concern in nerve guidance channel design and has the capacity to influence axonal growth rate [17–19]. Stiffness of neat GP fibers as compared to fibers treated with varying concentrations of GP was determined using atomic force spectroscopy. Crosslinking of fibers resulted in a significant increase in reduced elastic modulus from 87 ± 53 kPa for neat Cht fibers to 7.89 ± 2.53 MPa for Cht fibers that had been treated with 0.5% GP ($p < 0.01$) (Fig. 2B).

2.2.3. Surface hydrophilicity

GP treatment introduces extra hydrophilic groups to Cht nanofibers. We utilized the static sessile drop method to assess wettability of the scaffold surface. Cht nanofibers coated with poly-L-lysine (PLL) and laminin were tested in parallel for reference. The mean contact angle of non-coated Cht nanofibers was 83.8° ± 4.2°. Following treatment with 0.25% GP there was an increase in wettability as reflected upon by a reduction in contact angle to 58.6° ± 4.7°. In comparison, PLL/laminin-coated chitosan nanofibers demonstrated a contact angle of 18.2° ± 3.2°.

2.2.4. Susceptibility to swelling and lysozymal degradation

Given that neat Cht nanofibers are susceptible to swelling and dissolution *in vivo* [20], it is pertinent to assess whether genipin treatment helps to maintain surface topography. To assess for swelling, neat and 0.25% GP-treated Cht nanofibers were submerged in PBS at 37 °C for a period of 2-weeks. Initial morphology between the two groups was similar (Fig. 3A–B). By 2-weeks, swelling in neat nanofibers was evident, resulting in an increase in fiber diameter and blurring of edges (Fig. 3C). In contrast, fiber morphology was preserved in GP-treated fibers (Fig. 3D). Statistical comparison of fiber morphology between the two groups was achieved by utilizing the Canny method for edge detection (Supplementary Fig. 1) which confirmed that there was significant swelling in neat fibers only (Fig. 3E). To demonstrate resistance to degradation via enzymatic hydrolysis, neat and 0.25% GP-treated Cht nanofibers were incubated in lysozyme at 37 °C for two-weeks. Again, there was the tendency for neat Cht nanofibers to swell, fuse and lose their surface topography (Fig. 4A). In contrast, the topography of GP-treated nanofibers was preserved (Fig. 4B).

2.3. Effect of genipin treatment on neural cell cultures

2.3.1. Culture of Schwann cells on aligned nanofiber scaffolds

Schwann cells have a crucial role in facilitating peripheral nerve regeneration. Endogenous Schwann cells dedifferentiate and proliferate in response to injury, and switch on an axon-supportive program that promotes functional recovery [21]. Nerve guidance channels that are pre-seeded with sciatic nerve-derived Schwann cells [22] or bone marrow-derived Schwann cells [4] have shown promise in assisting post-traumatic nerve regeneration.

We seeded rat sciatic nerve-derived Schwann cells onto aligned electrospun Cht nanofibers to test for the capacity of varying substrata to support cell growth. Schwann cells were able to attach to and align along the main fiber axis when seeded upon both neat (Fig. 5A and D) and 0.5% genipin-treated fibers (Fig. 5B and E). In the absence of surface topography, Schwann cells seeded upon PLL/laminin-coated coverslips grew in a whorl-like pattern without evidence of directionality (Fig. 5C and F). The proportion of propidium iodide-stained apoptotic cells amongst Schwann cells established upon PLL/laminin, neat fibers and GP-Cht fibers amounted to 0.15 ± 0.11%, 3.94 ± 1.30% and 3.38 ± 0.77% respectively. The Ki67 proliferation indices amongst these conditions amounted to 37.9 ± 5.8%, 15.1 ± 2.8% and 26.9 ± 10.0%, and respectively (Fig. 5G–I). While Schwann cell growth was most favoured when cultured upon PLL/laminin coated coverslips, there was a tendency for GP treatment to increase proliferation and reduce apoptosis in comparison to culture on neat nanofibers, albeit not reaching statistical significance.

2.3.2. Culture of dorsal root ganglia explants upon aligned nanofiber scaffolds

Cultured dorsal root ganglia (DRG) explants were utilized as an *in vitro* model of peripheral nerve regeneration. After 5-days of culture in medium supplemented with nerve growth factor, Schwann cell migration and neurite length was compared between explants seeded upon neat Cht nanofibers, GP-Cht nanofibers, and uncoated coverslips. Immunocytochemistry against TUJ1 and S100β allowed for the identification of neurites and Schwann cells respectively.