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Purine-crosslinked injectable chitosan sponges promote oligodendrocyte progenitor cells' attachment and differentiation†

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Oligodendrocyte Progenitor Cells (OPCs) reside in the central nervous system (CNS) and are responsible for remyelinating axons after a spinal cord injury (SCI). However, the remyelination process is incomplete and abnormal due to the inability of OPCs to fully differentiate at the site of injury. In this study a newly developed injectable chitosan sponge crosslinked using guanosine 5′-diphosphate (GDP) was used to enhance OPC survival, attachment and differentiation. This purine-based biomaterial is the first of its kind and its inception was based on the growing body of literature concerning the role of purinergic signalling in the CNS. GDP-crosslinked chitosan sponges are rapidly-gelling and can be easily administered *in situ* using an injection system based on a double-lumen design. The chitosan sponges prompted OPC differentiation even in the presence of mitogens. Moreover, neurotrophin-3 (NT-3) was successfully entrapped in the sponges and a sustained release for up to 30 days was achieved. OPCs were shown to differentiate into mature oligodendrocytes that express myelin basic protein (MBP) when cultured on sponges containing NT-3. These findings, along with the suitable physicochemical and biological properties, make these sponges conducive to use as viable therapeutic agents for enhancing remyelination post-SCI.

Introduction

Oligodendrocyte Progenitor Cells (OPCs) play a central role in the remyelination process that takes place after a contusion spinal cord injury (SCI).[1] OPCs are recruited from both the white matter and the subventricular zone to remyelinate intact axons that have been demyelinated after the injury. However, remyelination that takes place is usually abnormal; myelin sheaths produced are thinner and have shorter internode segments.[2,3] The consensus in the literature is that failure to fully remyelinate is related to the failure of OPCs to fully differentiate at the site of injury.[3] Therefore, there remains a clinical need for a therapy that targets endogenous OPCs and improves their differentiation post-SCI.

Investigators exploring the use of biomaterials in the SCI field have been mainly concerned with promoting neuronal regeneration.[4] Even though this approach is critical, there is enough evidence to show that enhancing remyelination can also translate into functional recovery if it occurs during the early stages of injury.[5,6] Remyelination of intact axons that lost their myelin can prevent their degeneration after an injury and could limit neurological loss.[7] Preserving 5–10% of the axons after an SCI has been shown to re-instate some locomotor functions.[8,9] However, there have been few studies focused on developing biomaterials to specifically target remyelination post-SCI.[10–14]

We recently reported the fabrication and characterization of rapidly-gelling, injectable purine-based chitosan sponges. Their desirable physicochemical and biological properties make these sponges suitable for a wide range of tissue engineering and drug delivery applications, particularly for OPC attachment and differentiation.[15] Guanosine 5′-diphosphate (GDP) was used to ionically crosslink chitosan *via* phosphate–amine interactions, while guanosine's presence in the sponge composition was hypothesized to improve OPCs' attachment, survival and differentiation. The trophic effects of guanosine in the central nervous system have been widely investigated. Guanosine has been shown to have neuroprotective and oligodendrocyte-protective effects.[12,16,17] In addition, administering guanosine *in situ* enhanced remyelination post-SCI, although the exact mechanism of action remains elusive.[18–20] Interestingly, DNA aptamers rich in guanosine improved remyelination in a mouse model of multiple sclerosis.[21]

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Herein, OPCs were cultured on GDP-crosslinked chitosan sponges to investigate their maturation in both mitogenic and differentiation media. OPC differentiation was examined in mitogenic media to explore whether the chitosan sponges were able to induce differentiation even in the presence of mitogens. Moreover, washed and unwashed sponges were examined in order to explore whether neutralizing the sponges to pH 7 by thoroughly washing with media significantly affected OPC attachment and differentiation. To corroborate the results obtained from GDP-crosslinked chitosan sponges and the role played by GDP, OPC differentiation was also assessed in the presence of different GDP concentrations. Immunolabeling was used to evaluate OPCs' maturation in response to GDP-crosslinked chitosan sponges and different GDP concentrations. To further promote OPCs' differentiation, neurotrophin-3 (NT-3) was entrapped in the sponges and the release kinetics was assessed using ELISA. NT-3 has been shown to induce significant enhancement in remyelination post-SCI.[22] Moreover, NT-3 was shown to increase myelination *in vitro* as compared to controls.[23]

Through this study we demonstrate that injectable GDP-crosslinked chitosan sponges are capable of promoting OPC differentiation into mature myelin basic protein (MBP)-expressing oligodendrocytes, and thereby propose that purine-based chitosan sponges provide a promising platform for developing new therapies that can target remyelination post-SCI.

Experimental section

Materials

Chitosan (high molecular weight; degree of deacetylation >90%; viscosity of 3000 cp) was purchased from MP Biomedicals, LLC; guanosine 5′-diphosphate (GDP), poly-L-ornithine hydrobromide, sodium bicarbonate, bovine serum albumin, waymouth medium and human NT-3 ELISA kits were from Sigma Aldrich; hydrochloric acid (50% v/v) was from Labchem. All reagents used for the extraction, purification and immunostaining of OPCs have been previously reported.[24] Recombinant Human NT-3 was purchased from R&D Systems; collagen type I solution from rat tail (3.67 mg ml^{-1}) was from BD Biosciences; glass bottom microwell dishes (Ø = 35 mm, uncoated and γ-irradiated) were from MatTek Corporation; ClearMount mounting solution was from Invitrogen; and protein LoBind tubes were from Eppendorf.

OPC extraction, culturing and immunocytochemistry

OPCs were extracted and purified from brains of neonatal Sprague-Dawley rats as previously described.[25] Experiments were approved by the McGill animal care committee. OPCs were cultured on poly(ornithine)-coated coverslips in serum free media (SFM) consisting of a Dulbecco's Modified Eagle's Medium (DMEM)–F12 mixture (1 : 1), 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 0.1% bovine serum albumin (BSA), 25 μg ml^{-1} human transferrin, 30 nM triiodothyronine, 20 nM hydrocortisone, 20 nM progesterone, 10 nM biotin, 5 μg ml^{-1} insulin, 16 μg ml^{-1} putrescine, and 30 nM selenium. Under these conditions, progenitors divided, while astrocytes and microglia did not proliferate. Mitogenic media was prepared by supplementing SFM with 2.5 ng ml^{-1} of both platelet-derived growth factor ($PDGF_{AA}$) and basic fibroblast growth factor (bFGF), and differentiation media was prepared by removal of these mitogens (PDGF and bFGF) and the addition of 3% (v/v) calf bovine serum to SFM. SFM media was changed every two days unless mentioned otherwise.

For immunocytochemistry, sponges and coverslips were washed thoroughly with phosphate buffered saline (PBS) and incubated in horse serum for 15 minutes to prevent non-specific adsorption of antibodies to the sponges or coverslips' surface. A2B5 and/or GalC primary antibodies were added to the sponges and coverslips, and the samples were placed in a 37 °C incubator for 30 minutes. Samples were then washed twice with DMEM and fixed using 4% paraformaldehyde for 30 minutes. At this stage two different procedures were used depending on whether MBP staining was required or not. If MBP staining was not required, fluorescently-tagged secondary antibodies (GαM-IgM and GαM-IgG3) were applied to the samples for one hour followed by washing and nuclei staining using Hoechst. If MBP staining was required, the samples were incubated in a Triton X-100 solution (0.3%) for 30 minutes. Samples were then washed with PBS to rehydrate the cells and further incubated in 10% goat serum solution supplemented with 0.1% Triton X-100 and sodium azide to block and further permeabilize the cells. MBP primary antibody was diluted in the previously mentioned buffer and applied to the samples. The samples were incubated in the MBP primary antibody for 45 minutes and then washed thoroughly with PBS. The fluorescently-tagged secondary antibody (GαM-IgG2b) was again diluted in the same buffer and samples were incubated for one hour while covered with aluminium foil. Finally, samples were washed and nuclei were stained with Hoechst.

Fabrication of GDP-crosslinked chitosan sponges

Sponges were prepared as previously reported.[15] In brief, four formulations were prepared that had the acronyms C(X)PH(Y), where 'X' and 'Y' represent the chitosan concentration and pH of the chitosan solution respectively. The four sponge formulations were: C3PH5, C3PH6, C6PH5 and C6PH6. Prior to the preparation of the chitosan sponges, all Eppendorfs, utensils and glass vials were sterilized by placing under UV for 30 minutes. Chitosan was dissolved in 0.06 M hydrochloric acid (HCl) solutions to obtain the desired concentrations (*i.e.* 3 and 6 mg ml^{-1}). The solutions were mixed thoroughly until all chitosan was dissolved. Sodium bicarbonate (1 M solution) was added, 50 μl at a time, to the chitosan solutions until the desired pH was achieved (*i.e.* pH 5 and 6). The four formulations were then sterilized by filtration through sterile 0.45 μm syringe filters. GDP (100 mg ml^{-1}) was prepared by dissolving in deionized water. To fabricate the sponges, 1.7 ml from each formulation was placed in a 2 ml Eppendorf and supplemented with 0.3 ml of the GDP solution. The formed sponges were removed from the Eppendorf, placed in a 48 well

plate and, depending on the experiment, were either washed three times in DMEM or left unwashed in their respective supernatant solution.

NT-3 entrapment and release from chitosan sponges

NT-3 (0.1 μg) was added to 100 μl of sterile deionised water and the total amount of NT-3 was investigated for encapsulation. The chitosan solutions C3PH5 and C6PH6 were prepared as previously discussed. However, for this procedure 1.6 ml of the chitosan solutions (instead of 1.7 ml) were placed in Protein LoBind Eppendorfs and supplemented with 100 μl of NT-3 solution. GDP (0.3 ml) was then added to form the sponge as previously mentioned. The sponges were then removed and placed in another LoBind Eppendorf containing 0.5 ml of PBS with 0.1% BSA. The supernatant solutions left over from the sponge formation were centrifuged to pellet any sponge debris and an NT-3 ELISA kit was used to determine the concentration of free NT-3 in the solution. Eqn (1) was then used to calculate the entrapment efficiency, where W_{initial} is 0.1 μg, and W_{free} is the weight of free NT-3 for each preparation. Sponges containing NT-3 were placed in an incubator at 37 °C and under mild agitation. At each time point the release buffer (PBS + 0.1% BSA) was removed, frozen and replaced with a fresh solution. The release kinetics was assessed up to 30 days and the cumulative release was calculated using eqn (2), where W_t is the weight of NT-3 released at time t.

$$\text{EE}(\%) = \frac{W_{\text{initial}} - W_{\text{free}}}{W_{\text{initial}}} \times 100 \quad (1)$$

$$\text{CR}(\%) = \sum_{t=1}^{30} \left(\frac{W_t}{W_{\text{initial}}} \times 100 \right) \quad (2)$$

Surface characterization using SEM and XPS

In order to assess the morphology of the sponges, a Hitachi S-4700 Field Emission Scanning Electron Microscope (FE-SEM) at 2 keV and at a current of 10 μA was used to acquire images of the sponges. Moreover, the GDP availability on the surface of the sponges was determined using a Thermo Scientific X-ray photoelectron spectroscope (XPS) and by observing the P 2p peak using a high resolution scan.

Assessing OPC differentiation in the presence of GDP

OPCs (2×10^4 cells ml^{-1}) were cultured on Nunc™ plates (2D surface) for 24 hours followed by a media change to proliferation media to increase cell number. After 24 hours, the media was further changed to differentiation media. The differentiation media contained GDP at the following concentrations (1, 1.5, 2, 2.5, 3, 3.5 and 4 mM) plus the control (no GDP). The media was changed every other day and after 4 days in differentiation media, nuclei and GalC were stained with Hoechst and FITC respectively. A fluorescence plate reader, SpectraMax i3 from Molecular Devices, was used to read Hoechst and FITC fluorescence intensities of fixed cells at 37 evenly distributed points in each well. The average intensity was calculated per well and three wells per experimental group were used to calculate the overall relative fluorescence units ± SEM.

OPC differentiation on chitosan sponges

OPCs (2×10^4 cells ml^{-1}) were directly cultured on the four sponge formulations, either washed or unwashed, and poly-(ornithine)-coated coverslips (controls). After 24 hours, the media was replaced with mitogenic media and the samples were incubated in this media for 4 days. Immunolabeling was then performed to stain for progenitor (A2B5+) and differentiating (GalC+) OPCs. After immunostaining was complete, sponges were mounted on glass bottom microwell dishes and enough mounting solution was added to cover the whole sponge. Confocal microscopy was used to image 5 different areas (Z-stack images with a gap of 1.5 μm between each image) per sponge and the total number of nuclei per unit volume (since the sponge is a 3D structure) was counted using the 'ObjectCounter3D' plug-in on the ImageJ software. Cells expressing Hoechst co-localized with A2B5 or GalC were then counted and the percentages of progenitors and differentiating cells were calculated respectively.

Assessment of myelin basic protein expression on chitosan sponges

OPCs (2×10^4 cells ml^{-1}) were cultured on washed C3PH5 and C6PH6 sponges (optimum formulations based on previous experiments), with and without 0.1 μg of NT-3, for 8 days. The first four days were in mitogenic media and the following four days were in differentiation media. Both mitogenic and differentiation media were changed every 2 days. Type I rat tail collagen gels were used as three-dimensional controls instead of the two-dimensional poly(ornithine)-coated coverslips. Collagen was chosen since it has been recently reported to support OPC adhesion and has been widely investigated in many tissue engineering applications.[26] For the preparation of collagen gels, first a neutralization solution was prepared by mixing 10× waymouth and 0.34 N NaOH solutions in a ratio of 3 : 2 respectively. This neutralization solution (300 μl) was then added to 1 ml of collagen type I solution, mixed thoroughly and was left to gel in the incubator for 15 minutes. Confocal microscopy was again used to calculate the percentage of differentiating OPCs (GalC+/MBP−) and mature oligodendrocytes (GalC+/MBP+) and the total differentiation for each sponge was calculated by the summation of differentiating and mature oligodendrocytes. It is important to mention that quantification was done by counting the number of OPCs expressing the different markers and normalizing to the total number of cells per unit volume of sponge.

Examining the effect of chitosan sponges on PDGF availability in the media

Washed C3PH5, C3PH6, C6PH5 and C6PH6 sponges were placed in 0.5 ml of SFM containing bFGF and PDGF for 4 days (no cells). Poly(ornithine)-coated coverslips (controls) were also immersed in the same media for 4 days. At day 1 and day 4, 10 μl of media from each well was removed, placed in

Protein LoBind Eppendorfs, diluted 20 times in PBS and frozen for later analysis. Once all samples were collected, an ELISA kit was used to measure the concentrations of PDGF in the media.

Statistical analysis

Experiments were repeated three independent times with triplicates in each experiment. Prior to any statistical analysis a Shapiro–Wilk normality test was conducted on the data. If the data were not normally distributed, then a non-parametric Kruskal–Wallis ANOVA was used to compare the significant difference between the different groups. If the data were normally distributed, a one-way ANOVA was used to assess the statistical difference.

Results

Injectability and surface characterization of GDP-crosslinked chitosan sponges

GDP crosslinks chitosan chains very rapidly (t_{gel} < 1.6 seconds),[15] and therefore in order to avoid clogging of the needle during *in situ* administration, a double-barrel syringe with a mounted double-lumen needle system for mixing GDP and chitosan solutions at the tip of the needle was engineered. This injection system provided an outer flow of chitosan and an inner flow of GDP within the needle, which mixed only at the tip during injection (Fig. 1A and B). SEM images revealed a micro-architecture that is highly porous with interconnected pores (Fig. 1C). A highly porous structure is desirable for achieving drug delivery *via* diffusion. Moreover, XPS analysis of the sponges showed the presence of phosphorus, which indicates that GDP is present on the surface and can interact with OPCs (Fig. 1D).

OPC differentiation in the presence of GDP and on GDP-crosslinked sponges

The effect of various GDP concentrations on OPC differentiation is presented in Fig. 2. Results demonstrated that OPCs exposed to different concentrations of GDP did not undergo proliferation as readily as controls and therefore the fluorescence intensity produced by the nuclei was lower than the controls. However, there was no significant difference in the fluorescence intensity of GalC between GDP-treated OPCs and controls. Therefore, normalizing GalC to the number of cells demonstrated an increased differentiation per cell in the presence of GDP.

Conversely, OPCs adhered to the GDP-crosslinked chitosan sponges. Progenitors (A2B5+) acquired either a bipolar morphology or a stellate morphology with few short processes. Meanwhile, differentiating GalC$^+$ OPCs had multiple processes (Fig. 3A). In some areas on the sponge, where OPCs were in close proximity, differentiation was much more pronounced (Fig. 3B). Results from this experiment demonstrated that the percent of GalC+ cells on washed C3PH5 (28.7 ± 6.5%), C6PH5 (21.3 ± 4.8%) and C6PH6 (24.1 ± 3.4%) sponges was signifi-

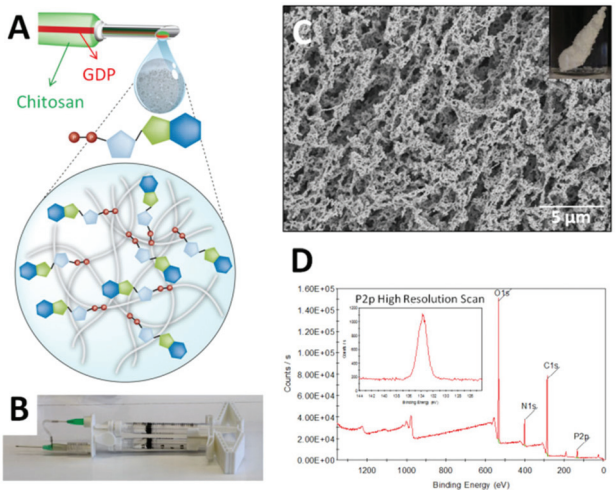

Fig. 1 (A) Schematic representation of the method of injection using a double-lumen needle system. First, mixing of the two solutions takes place at the tip of the needle and results in rapid sponge formation, which precipitates out of the solution. This gelation occurs due to the ionic crosslinking of chitosan chains *via* the phosphate groups in GDP; (B) the double-barrel, double-lumen injection system used to administer the sponge *in situ*; (C) SEM image of the chitosan sponge (C6PH6) and a photograph of the formed sponge; and (D) a survey XPS spectrum of the chitosan sponge C6PH6 illustrating the expected carbon, oxygen and nitrogen peaks pertaining to chitosan, and the phosphorus peak associated with GDP. The top left spectrum is a high-resolution scan of the phosphorus peak. The presence of phosphorus confirms the presence of GDP on the surface of the sponge.

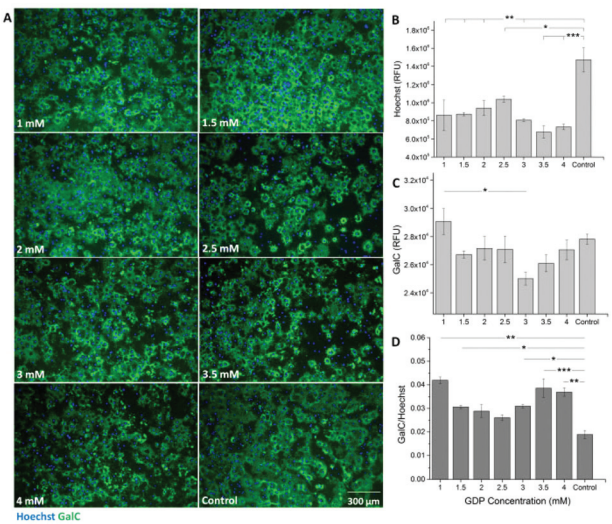

Fig. 2 OPC differentiation in response to different concentrations of GDP. (A) Fluorescence microscopy images of GalC+ oligodendrocytes after exposure to different concentrations of GDP; (B) quantification of relative fluorescence units (RFU) of nuclei (Hoechst staining) measured at an excitation of 343 nm and an emission of 483 nm; (C) quantification of GalC (FITC staining) measured at an excitation of 495 nm and an emission of 519 nm; (D) normalizing GalC to Hoechst as an assessment of differentiation. Values represent mean ± SEM (n = 3, *P < 0.05, **P < 0.01, ***P < 0.001).

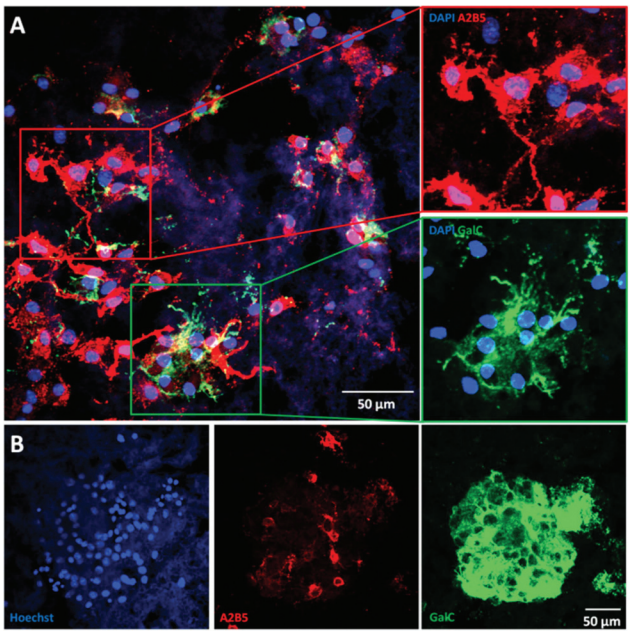

Fig. 3 OPC morphology on GDP-crosslinked chitosan sponges after culturing in mitogenic media for 4 days. (A) Confocal images of OPCs (A2B5+) and differentiating OPCs (GalC+) cultured on unwashed C3PH6. Progenitors acquired a bipolar morphology and extended few processes, while differentiating cells (GalC+) had more processes similar to their endogenous morphology; (B) a confocal image of OPCs cultured on washed C3PH5 demonstrating the pronounced differentiation of OPCs when they are in close proximity.

cantly higher than the controls (9.0 ± 3.8%) (Fig. 4). Moreover, all unwashed sponges (C3PH5: 16.1 ± 2.57%; C3PH6: 22.68 ± 3.31%; C6PH5: 22.2 ± 2%; C6PH6: 17.35 ± 1.88%) showed a significant increase in OPC differentiation compared to the controls. This increase in differentiation on the sponges was also confirmed by the significantly lower ($P < 0.001$) percentage of progenitor cells (A2B5+) on the sponges as compared to controls.

Effect of chitosan sponges on PDGF availability in the media

The effect of sponges on PDGF availability in the media was investigated to ensure that the sponges did not absorb PDGF and deplete its availability in the media and, in turn, inhibit its proliferative effect on OPCs. Surprisingly, measuring the PDGF concentrations in the presence of the sponges and poly(ornithine)-coated coverslips (control) with no OPCs after 1 and 4 days demonstrated that the PDGF concentration was significantly higher in the presence of the sponges as compared to the control (Fig. S1†). This preserving effect of the sponges on PDGF was attributed to the slightly acidic environment produced by the sponges that maintained the bioactivity of PDGF. This confirmed that OPC differentiation was not due to PDGF depletion in the media. Moreover, since bFGF has a similar molecular weight and isoelectric point to PDGF, one can also expect that the sponges will not deplete their presence in the media.

NT-3 entrapment and effect of its release on MBP expression

The entrapment efficiencies of NT-3 were found to be 73.7 ± 1.88% and 74.1 ± 0.350% in C3PH5 and C6PH6 respectively. Moreover, the release kinetics demonstrated controlled release of NT-3, where 27.2 ± 1.3% and 20.9 ± 2.1% of entrapped NT-3 was released from C3PH5 and C6PH6 respectively over a period of 30 days (Fig. 5). Release from C3PH5 was significantly higher than that from C6PH6 from day 3 onwards due to the higher water retention (swelling) capability of C3PH5 as compared to C6PH6, which allows more NT-3 to be released *via* diffusion.[15]

OPCs cultured on C3PH5 and C6PH6, with and without NT-3, for 8 days differentiated to oligodendrocytes. Mature oligodendrocytes expressed MBP on both sponge formulations and collagen controls (Fig. 6A). Results demonstrated a significant increase in total differentiation on C3PH5 (52.5 ± 2.68%), C3PH5 + NT-3 (57.1 ± 3.65%) and C6PH6 + NT-3 (56.3 ± 3.25%) as compared to the collagen controls (39.7 ± 3.30) (Fig. 6B). Further analysis indicated that there was no significant difference in %GalC+/MBP− OPCs amongst all the experimental groups. However, there were significantly more mature oligodendrocytes (GalC+/MBP+) on C3PH5 and C3PH5 + NT-3 as compared to the controls. The incorporation of NT-3 into the sponges was shown to enhance the number of differentiating cells; however, it had no effect on OPC proliferation (Fig. 6B and C). Interestingly, the total cell density was significantly higher on collagen gels as compared to the chitosan sponges (Fig. 6C). This demonstrated that collagen gels encouraged more OPC proliferation compared to the chitosan sponges, while the latter induced more OPC differentiation. This was evident from the presence of many dividing cells on the collagen gels as indicated by arrows in Fig. 6A. Also, there was no statistical difference in total cell density between C3PH5 and C6PH6 sponges (±NT-3) even though C3PH5 had slightly higher cell densities compared to C6PH6.

Discussion

Various cell transplantation strategies have been employed to generate new oligodendrocytes post-SCI and improve neurological function.[5] However, since OPCs are readily available *in situ*, other therapies were implemented to improve endogenous OPCs' differentiation post-SCI.[12] The strategy adopted in this study was an inductive tissue engineering one, where the biomaterial was designed to provide a suitable microenvironment with specific cues to promote the differentiation of endogenous OPCs.

In this study, differentiation of primary rat OPCs was investigated on a purine-based biomaterial fabricated using chitosan and GDP. GDP served two purposes, namely to ionically crosslink chitosan through the phosphate–amine interaction and to promote OPC differentiation through the presence of guanosine in the sponge composition. The rationale behind the sponge composition was based on purinergic signaling studies that demonstrated the effectiveness of guanosine in

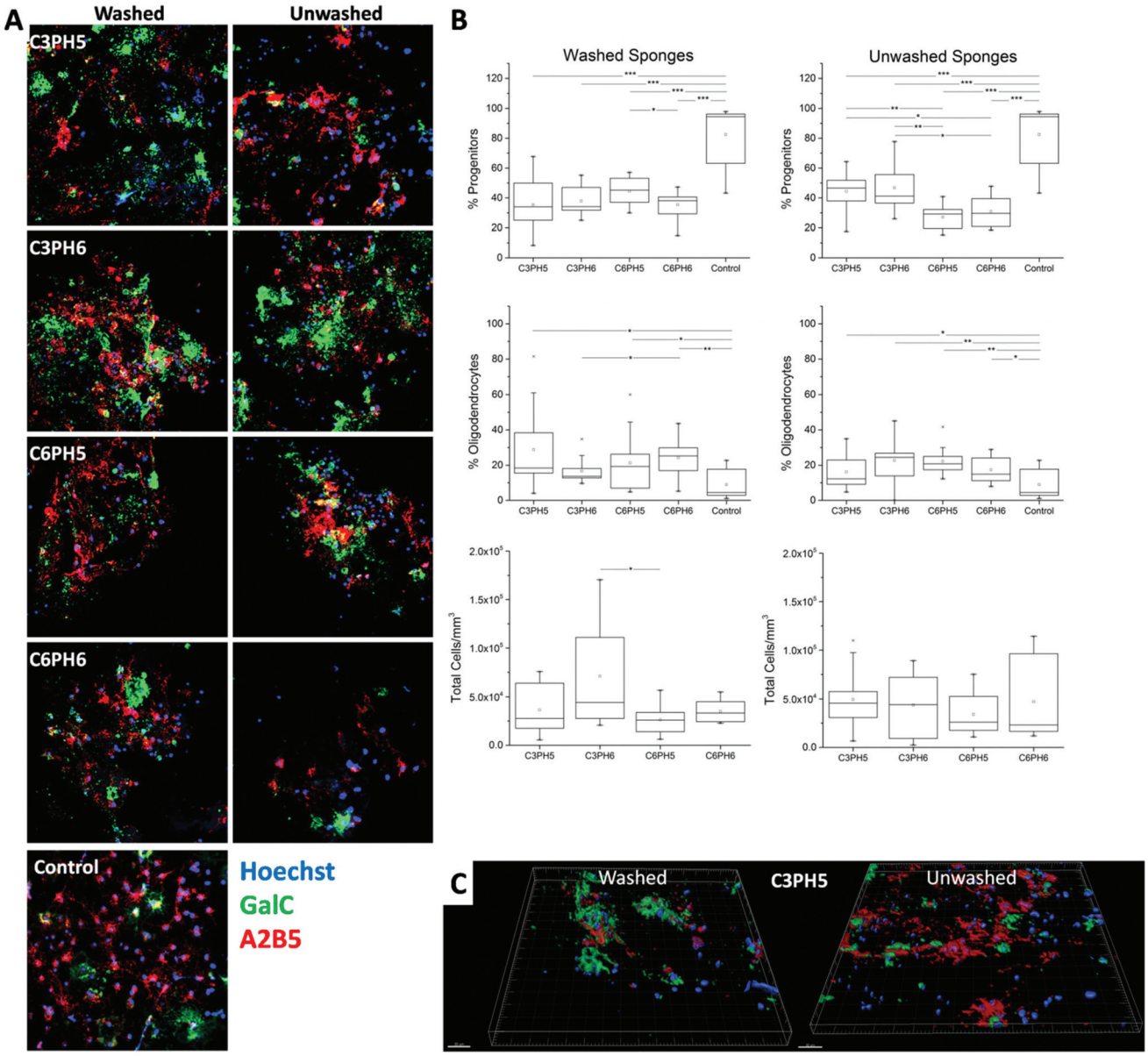

Fig. 4 Assessment of OPC attachment and differentiation on chitosan sponges in mitogenic media. (A) Confocal images of OPCs cultured on the four washed and unwashed sponge formulations and poly(ornithine)-coated coverslips (controls) for 4 days in the presence of mitogens (PDGF and bFGF). OPCs were stained for the progenitor marker A2B5, the pre-oligodendrocyte marker GalC and nuclei; (B) box and whiskers plots demonstrating the percent of progenitors, percent of oligodendrocytes and total cell density (cells mm^{-3}) in both washed and unwashed sponges; (C) a three dimensional rendering of OPCs cultured on washed and unwashed sponges demonstrating the spreading of OPCs on the sponge. Values represent mean ± SEM (n = 3, *P < 0.05, **P < 0.01, ***P < 0.001).

enhancing remyelination *in situ* post-SCI. Jiang *et al*. discovered that guanosine administered intraperitoneally during the chronic stage of SCI improved functional locomotor recovery partly by increasing the number of mature oligodendrocytes at the site of injury.[18] The same group also demonstrated that administration of guanosine significantly enhanced the proliferation of OPCs at the site of injury, and enabled their differentiation into mature oligodendrocytes.[20] Whether guanosine or its hydrolyzed component guanine is responsible for the enhanced remyelination process remains elusive.[27] Guanosine has also been shown to stimulate astrocytes to release nerve growth factor (NGF) and transforming growth factor β (TGF-β) through either low affinity binding to A1/A2 purinergic adenosine receptors or by acting directly on a guanosine-specific cell surface receptor.[27,28]

In a broader sense, the role of purinergic signaling in the central nervous system has been gaining wide interest in the past three decades.[29] The discovery that glial cells (oligodendrocytes, astrocytes and microglia) express purinergic receptors that respond to extracellular purines has been changing

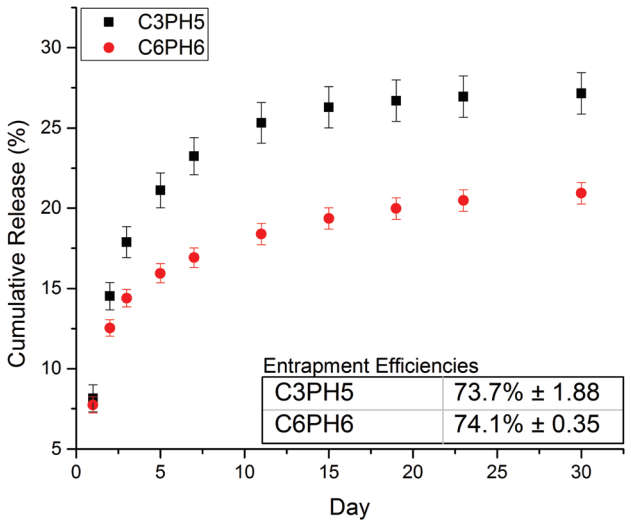

Fig. 5 Entrapment efficiencies and cumulative release of NT-3 over 30 days from C3PH5 and C6PH6 sponges measured at 37 °C and under mild agitation. Values represent mean ± SEM (n = 3).

our understanding of neuron–glia interactions and myelination in the central nervous system.[29] Adenosine, for example, is released by firing axons and has been shown to act on adenosine receptors in OPCs to inhibit their proliferation and promote their differentiation and myelin formation.[30] Interestingly, it has also been shown that extracellular guanosine can act partly through adenosine receptors.[31] Guanosine was shown to promote neurite outgrowth and provide neuroprotective and oligodendrocyte-protective effects during hypoxia, hypoglycemia and excitotoxicity.[27,31] Therefore, envisioning and designing new purine-based biomaterials can herald a new era of therapeutics that can target various neurological traumas and diseases including SCI.

Indeed, it is demonstrated in this study that OPCs cultured on the sponges adhered to the sponges and acquired physiological phenotypes. This was in contrast to OPCs cultured on pure chitosan films (results not shown), where attachment was minimal and no cellular processes were observed. The three-dimensional micro-architecture and the presence of GDP significantly improved OPC attachment on the sponges. This is

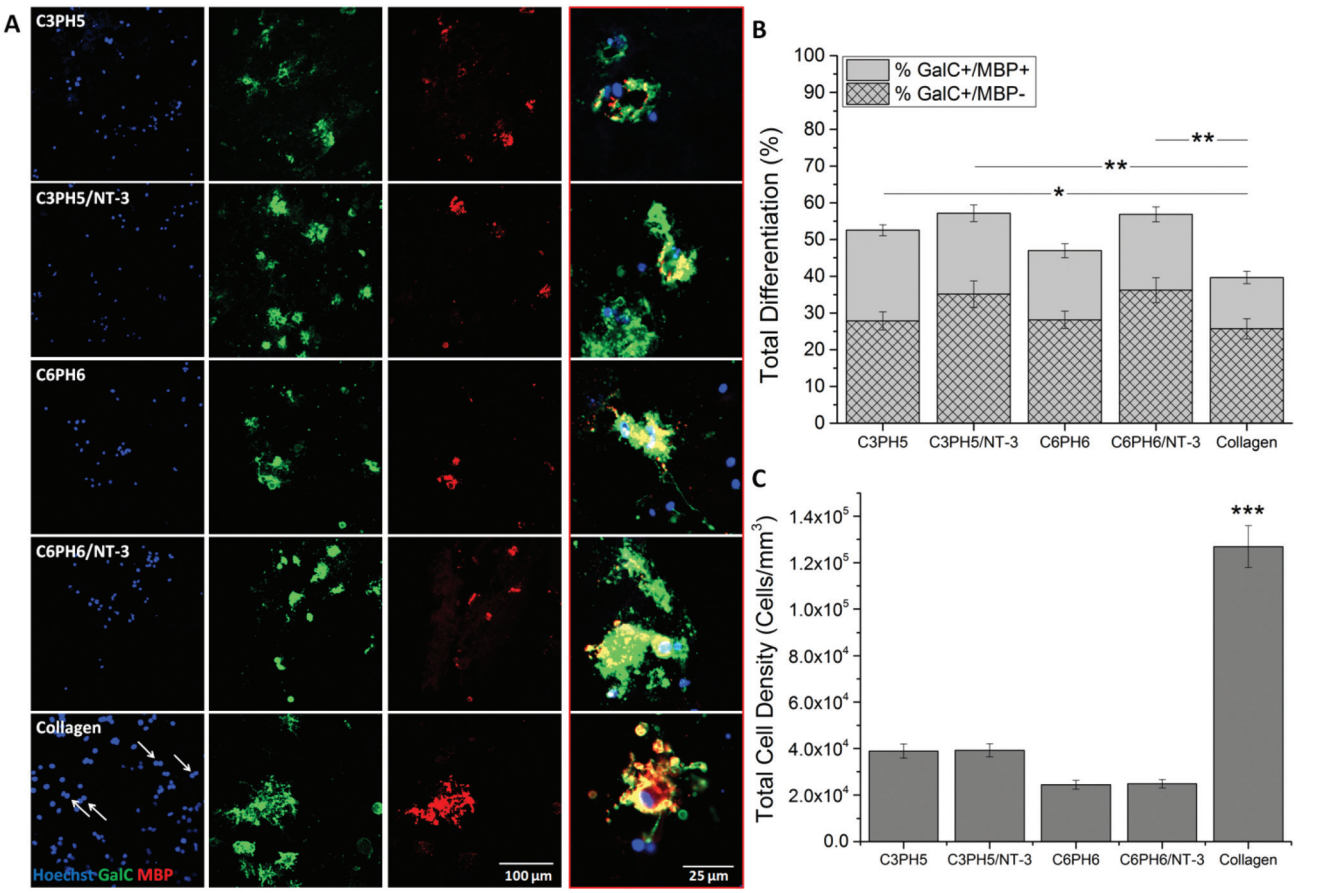

Fig. 6 Assessment of OPC differentiation to mature oligodendrocytes (GalC+/MBP+) on C3PH5 and C6PH6 (±0.1 µg of NT-3), and collagen gels (control). (A) Confocal images illustrating the nuclei, GalC and MBP staining; the last column of images is a higher magnification to illustrate the co-expression of MBP and GalC in mature oligodendrocytes (note: overlap of stains appears in yellow); (B) total number of cells that are differentiating as shown by a stack bar graph of %GalC+/MBP– and GalC+/MBP+ oligodendrocytes for each experimental group; (C) total cell density (cells mm^{-3}) on each experimental group; collagen gels had significantly higher cell density than all other groups. Values represent mean ± SEM (n = 3, *P < 0.05, **P < 0.01, ***P < 0.001).

not unusual, since previous studies have shown that nanotopography and porosity are just as critical as surface chemistry in determining stem cell attachment, morphology and differentiation.[32] Interestingly, NeuroGel™, a poly[*N*-(2-hydroxypropyl)-methacrylamide] hydrogel, has a similar nanotopography to the chitosan sponges, and was shown to support axonal growth and promote spinal cord repair.[33] Moreover, the confinement of GDP within the chitosan sponge had a pronounced effect on OPC differentiation. OPCs cultured on the chitosan sponges were able to differentiate readily even in the presence of mitogens. This phenomenon is again not unusual, since it has been previously shown that a differentiation factor can over-ride the proliferative effects of mitogens on OPCs and promote their differentiation.[30] Therefore, it is strongly believed that the GDP-crosslinked chitosan sponges can enhance OPC differentiation through similar mechanisms. Moreover, this enhanced differentiation was not dependent on whether the sponges were thoroughly washed or not.

Although the presence of GDP in the sponge might be sufficient to promote differentiation, the use of an injectable system provides the added advantage of entrapping neurotrophic factors and providing sustained release to further enhance remyelination. Moreover, it has been reported that some purines elicit their trophic effects in synergy with neurotrophic factors.[34] Rapid gelation ensured the high entrapment efficiency of NT-3, while the sustained release over 30 days was attributed to the highly porous micro-architecture of the sponge. The bioactivity of released NT-3 was evident from the enhanced maturation of OPCs cultured on sponges containing NT-3. More specifically, C3PH5 had the greatest effect on oligodendrocyte maturation and would be the most promising candidate for further exploration *in situ*.

Assessment of the effect of GDP on OPCs indicated that GDP (or its hydrolyzed component guanosine) promotes OPC differentiation. This was evident from the higher GalC production per cell in the presence of GDP as compared to the control. This is an interesting finding, since the effect of GDP on OPCs has not been previously reported. However, it has been reported that extracellular adenosine inhibits OPC proliferation and promotes their differentiation,[30] an effect that can also be caused by GDP (or guanosine), especially given that guanosine was shown to act on the adenosine A2 receptors.[27]

Finally, in addition to the enhanced differentiation observed, GDP-crosslinked chitosan sponges possess many other advantages over current biomaterials used for the treatment of SCI. Firstly, only a few injectable chitosan-based hydrogels have been explored for spinal cord regeneration in the literature,[35,36] and secondly, even fewer biomaterials were designed to target remyelination.[10] The injectability of the sponge makes it highly desirable in a clinical setting, since it can be administered into the injury site using minimally invasive surgery. Moreover, the rapid gelation ensures: sponge localization at the site of injection, prevention of unwanted diffusion into the surrounding tissue, and high entrapment efficiencies of bioactive molecules.

Conclusion

GDP-crosslinked chitosan sponges presented in this study are promising purine-based biomaterials that can be used as clinically-relevant therapies for enhancing remyelination post-SCI. Indeed, we demonstrated that this purine-based injectable sponge supported OPC survival, adhesion, and enhanced their differentiation to mature oligodendrocytes. Moreover, they featured a high entrapment efficiency and release of neurotrophic factors in a controlled manner. According to these findings, one can explore a higher concentration of NT-3 in the sponges, along with other neurotrophic factors such as brain derived neurotrophic factor (BDNF) and insulin-like growth factor 1 (IGF-1) to further enhance OPC differentiation. More interestingly, utilizing knowledge from the growing field of purinergic signalling, and exploring other purines such as guanosine tri-phosphate (GTP), adenosine di-phosphate (ADP) and adenosine tri-phosphate (ATP) to fabricate different purine-based chitosan sponges can yield promising therapies for application in the CNS.

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