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GENE DELIVERY

Polysaccharide-modified scaffolds for controlled lentivirus delivery *in vitro* and after spinal cord injury

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ABSTRACT

Gene delivering biomaterials have increasingly been employed to modulate the cellular microenvironment to promote tissue regeneration, yet low transduction efficiency has been a persistent challenge for *in vivo* applications. In this report, we investigated the surface modification of poly(lactide-co-glycolide) (PLG) scaffolds with polysaccharides, which have been implicated in binding lentivirus but have not been used for delivery. Chitosan was directly conjugated onto PLG scaffolds, whereas heparin and hyaluronan were indirectly conjugated onto PLG scaffolds with multi-amine crosslinkers. The addition of chitosan and heparin onto PLG promoted the association of lentivirus to these scaffolds and enhanced their transduction efficiency *in vitro* relative to hyaluronan-conjugated and control scaffolds that had limited lentivirus association and transduction. Transduction efficiency *in vitro* was increased partly due to an enhanced retention of virus on the scaffold as well as an extended half-life of viral activity. Transduction efficiency was also evaluated *in vivo* using porous, multiple channel PLG bridges that delivered lentivirus to the injured mouse spinal cord. Transgene expression persisted for weeks after implantation, and was able to enhance axon growth and myelination. These studies support gene-delivering PLG scaffolds for *in vivo* regenerative medicine applications.

1. Introduction

Biomaterial scaffolds are a versatile tool to create a permissive cellular microenvironment for regenerative medicine. Their tailorable architecture provides a structural niche for the engraftment of transplanted cells and the infiltration of endogenous progenitor cells. Functionalizing these scaffolds with viral vectors can expand their bioactivity, with the material providing a platform for maintaining vectors locally to obtain robust, prolonged expression of the transgene, which can either promote regeneration or counteract its inhibitors [1,2]. Strategies utilizing viral vectors permit a ready exchange of target genes, or delivery of multiple vectors, without altering the delivery system, as the vectors have similar physical properties independent of the gene sequence. Still, the transduction efficiency of viral vectors *in vivo* has been low historically, motivating the exploration of strategies that promote the retention or immobilization of viruses onto the biomaterial [3–10].

More recently, biomaterials have been designed to more efficiently incorporate and retain viral vectors [3–5]. In hydrogels, the physical properties such as mesh size, hydrophilicity, and degradation, dictate whether a vector is released or retained within the material [3,6,7]. In contrast, for macro-porous scaffolds, the fabrication procedures often diminish virus activity, which precludes the entrapment of vectors. Adding virus-affinity moieties to these biomaterial scaffolds further enhanced viral vector retention and led to localized transgene expression; however, the incorporation of these moieties can alter the biomaterial's properties [3,5], limiting their applications. As one approach, the envelope proteins of viral vectors were engineered to contain antibody-binding fusion proteins [8,9] or oligo-peptide tags and ligands [10] in order to control their association to materials. However, altering the virus coat can be time consuming and may reduce the virus transduction efficiency compared to the wild-type virus. Our strategy modifies the surfaces of biomaterials in order to promote interactions that can retain the vector while minimizing changes to the bulk properties.

Herein, we investigated the immobilization and controlled release of lentivirus on poly(lactide-co-glycolide) (PLG) scaffolds modified with polysaccharides post-fabrication. The polysaccharides employed in this study—heparin, hyaluronan, and chitosan—are naturally-derived polymers that have previously been incorporated onto PLG for regenerative medicine applications [11–19]. More recently, heparin has been

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utilized to immobilize and concentrate lentivirus for enhanced production efficiency [20], and this report extends this observation of binding to promote localized gene delivery. Heparin, hyaluronan and chitosan were immobilized onto the surface of porous PLG scaffolds using *N*-ethyl-*N*′-(3-(dimethylamino)propyl)carbodiimide/*N*-hydroxysuccinimide (EDC/NHS) chemistry that allows polysaccharides to be coupled to PLG without altering the bulk properties of the scaffold. Scaffolds modified with polysaccharides using this approach were then characterized for incorporation and retention of lentivirus on macro-porous structures, enhanced transgene expression both *in vitro* and *in vivo*, and evaluated for their ability to enhance tissue regeneration in a spinal cord injury model.

2. Materials and methods

2.1. Surface-modified PLG scaffolds and bridges

To form a 5 mm disk-shaped scaffold, a 1:30 ratio of PLG (75:25 ratio of D,L-lactide to L-glycolide, inherent viscosity: 0.76 dL/g; Lakeshore Biomaterials, Birmingham, AL) and salt (250–425 μm) was mixed using wet granulation, mechanically pressed at 1000 psi, equilibrated under 800 psi of carbon dioxide for 16 h and then released at 60 psi/min to foam into the final structure as previously described [5]. The scaffolds were leached overnight in distilled water. To form a 90% porous bridge for a mouse spinal cord hemisection lesion, a 1:1 PLG and salt (63–106 μm) mixture coated cylindrical sugar fibers drawn from 103 °C caramelized sucrose, was packed into a mold, was equilibrated under 800 psi of carbon dioxide for 16 h and was then released at 60 psi/min, as established previously [21]. The bridges were leached 30 min in distilled water. All materials were from Fisher Scientific unless otherwise indicated.

Scaffolds and bridges were modified by first incorporating the crosslinkers (1,6-diamino hexane (HDA, 1.5 mg in 10 μL isopropanol, which represents the solubility limit for HDA and is comparable to previous reports [11]), or chitosan (2.5–250 μg in 10 μL 2% acetic acid, 8183 mol. wt.)) onto the surface and then immersing the scaffold for 4 h into 1 mL of 1 M MES buffer in the presence of 9 mg EDC (CreoSalus Inc., Louisville, KY) and 6 mg NHS (Research Organics, Cleveland, OH). Heparin (180 USP/mg, ~16,000 Da [22]) and hyaluronan (Genzyme, 1330 Da, Cambridge, MA) were attached onto the crosslinker modified PLG by incorporating 2.5–250 μg of these polysaccharides in 10 μL MES buffer onto the crosslinker-modified scaffolds and then immersing the scaffold into the above EDC/NHS in MES solution overnight. The modified scaffolds and bridges were washed with distilled water and then dried until use.

The surface of the scaffolds was imaged using the Leo Gemini 1525 (Zentrum für Werkstoffanalytik Lauf, Pegnitz, Germany) at 10 kV after coating with osmium tetroxide. Conjugation of polysaccharides onto PLG was quantified by immersing the modified scaffolds for 3 h in dyes known to associate with them and then measuring the absorbance extinction of the solution. Chitosan was quantified using the extinction of Orange II at 480 nm, heparin using toluidine blue (Fisher Scientific) at 610 nm, and hyaluronan using alcian blue at 620 nm. Dye solutions were made according to previously established methods [23–25]. All materials are from Sigma-Aldrich unless otherwise indicated.

2.2. Lentivirus production and association to PLG scaffolds

Human embryonic kidney cells (HEK 293T) cells were cultured in Dulbecco's modified eagle medium supplemented with 10% fetal bovine serum and 1% penicillin–streptomycin. All cell culture materials were purchased from Invitrogen. Lentivirus was prepared using a previously established technique [3–7]. These cells were co-transfected with plenti-CMV-luciferase, plenti-CMV-sonic-hedgehog or plenti-UbC-beta-galactoside encoding vectors and the packaging vectors: pMDL-GagPol, pRSV-Rev, pIVSVSV-G using Lipofectamine 2000 (Roche Biosciences, Palo Alto, CA, USA). Supernatant was collected after 48 h, concentrated in PEG-it (SystemBiosciences, Mountain View, CA, USA) for 24 h, precipitated using ultracentrifugation and resuspended in PBS. Titer was determined using a qPCR lentivirus titer kit (Applied Biological Materials, Inc., Richmond, BC, Canada).

Virus (4×10^7 particles) was pipetted onto the scaffold to assess lentivirus incorporation and retention on the PLG scaffold, and these quantities are similar to those previously reported [4,5]. After a 10-minute incubation, the scaffolds were placed into a 96-well plate and washed with PBS (Sigma-Aldrich) to remove unassociated virus. The supernatant was titered using the qPCR kit. The scaffolds were placed into another 96-well plate, incubated in PBS at 20 °C, and removed after 1–3 days. The supernatant was again titered using the qPCR kit.

2.3. Lentivirus transduction in vitro

Transduction efficiency and proliferation were assessed qualitatively. Virus (4×10^7 particles) encoding for beta-galactosidase was pipetted onto the scaffold and washed with PBS after a 10-minute incubation, consistent with previous reports [4,5]. HEK 293T cells (1×10^6) were seeded onto the scaffold in a 6-well plate, similar to previous reports [4,5]. After 4 h, the scaffold was transferred into another 6-well plate and cultured for 3 days. To view transduction, scaffolds were fixed in 4% paraformaldehyde, incubated with 5-bromo-4-chloro-indolyl-β-D-galactopyranoside (X-gal) (Inalco SP, Milano, Italy) for 16 h and imaged using light microscopy (Leica Microsystems, Wetzlar, Germany). To assess non-transduced cells, scaffolds were counterstained with Nuclear Fast Red (Trevigan, Gaithersburg, MD).

To quantitatively assess transduction efficiency, virus (4×10^7 particles) encoding for luciferase was pipetted and dried onto the scaffold and washed with PBS. The scaffolds were incubated in PBS for 0 to 2 days at 20 °C, with 5×10^4 or 1×10^5 HEK 293 T cells subsequently seeded onto a scaffold in a 96-well plate (BD Falcon), consistent with previous reports [3,6,7]. After 4 h, the scaffold was transferred into a black 96-well plate and cultured for 3 days. Bioluminescence was assessed using the IVIS imaging system (Caliper, Hopkinton, MA, USA) as described previously [26]. The scaffolds were incubated with 50 mM of D-luciferin (Molecular Therapeutics, Inc., MI) for 4 h and imaged with two 10-second intervals.

2.4. Cell infiltration, gene expression in vivo

Gene expression was assessed following lentivirus delivery in the injured spinal cord. To assess transduction, virus (2.8×10^7 particles) was pipetted and dried onto a porous, 7-channel PLG bridge and stored at −80 °C until use. Animals were treated according the Animal Care and use Committee guidelines at Northwestern University. Surgery was performed as previously described ($n = 4$ per scaffold and time-point) [2,5,26]. C57Bl6 female mice (20 g, Charles River) were anesthetized using isoflurane (2%). A laminectomy was performed at T9–T10 for bridge implantation into a hemisection lesion that is 2.25 mm in length. The injury site was stabilized by suturing the muscles together and stapling the skin after covering the site with Gelfoam. Baytril (enrofloxacin 2.5 mg/kg SC, once a day for 2 weeks), buprenorphine (0.01 mg/kg SC, twice a day for 3 days), and lactate ringer solution (5 mL/100 g, once a day for 5 days) were administered post-operatively. Bladders were expressed twice daily until function recovered.

To quantify luciferase expression, mice were injected intraperitoneally with 150 mg/kg body weight of D-Luciferin (Molecular Therapeutics, Inc., MI). Bioluminescence was immediately assessed using the IVIS imaging system (Caliper, Hopkinton, MA, USA) as described previously [3–7]. The animals were imaged at 5 minute intervals until peak expression was confirmed. The ability of lentivirus delivering bridges to promote regeneration of axons after spinal cord injury was investigated by loading 7.0×10^6 particles of sonic hedgehog- or luciferase-

encoding virus onto the PLG bridge, which were implanted and retrieved after 8 weeks.

To characterize cell infiltration and gene transduction *in vivo*, explanted bridges were frozen and sectioned transversally in 18 μm thick slices. To visualize overall cell presence, sections were fixed in 4% paraformaldehyde, stained with eoisin, counter-stained with Mayer's hematoxylin (Surgipath Medical Industries), and imaged using light microscopy (Leica Microsystems, Wetzlar, Germany). To visualize axon extension and myelination, these sections were double stained with neurofilament-160 (NFM, Millipore, MAB1621; 1:1000) and myelin basic protein (MBP, Santa Cruz Biotech, sc-13914; 1:1000) with AlexaFluor 488 donkey anti-goat IgG (1:1000) and 555 donkey anti-mouse IgG (1:1000) secondaries and imaged using fluorescence microscopy (Leica Microsystems, Wetzlar, Germany). To visualize transduced cells, these sections were stained with anti-luciferase (1:50 dilution; Promega, Madison, WI) as the primary antibody and anti-donkey Dylight 549 as the secondary (1:250; Jackson ImmunoResearch, Westgrove, PA), and counterstained with Hoescht 33342 (Invitrogen). To assess the source of myelination, a triple stain of NFM (1:250), MBP (1:1000) and P0 (Aves Labs, mpz; 1:500) was performed with AlexaFluor 555 donkey anti-goat IgG (1:500), 647 donkey anti-mouse IgG (1:500), and 488 goat anti-chick IgY (Aves Labs, F-1005; 1:500) as secondaries.

2.5. Statistics

Single comparisons were analyzed using a Student t-test. Multiple comparisons pairs were analyzed using a one-way or two-way ANOVA with a Bonferonni post-hoc test as appropriate. For *in vivo* studies, a log 2 transform of the data was employed as described previously [27] using ANOVA with Bonferonni in order to account for unequal variance amongst expression levels. Significance was defined at a level of $p < 0.05$ unless otherwise indicated.

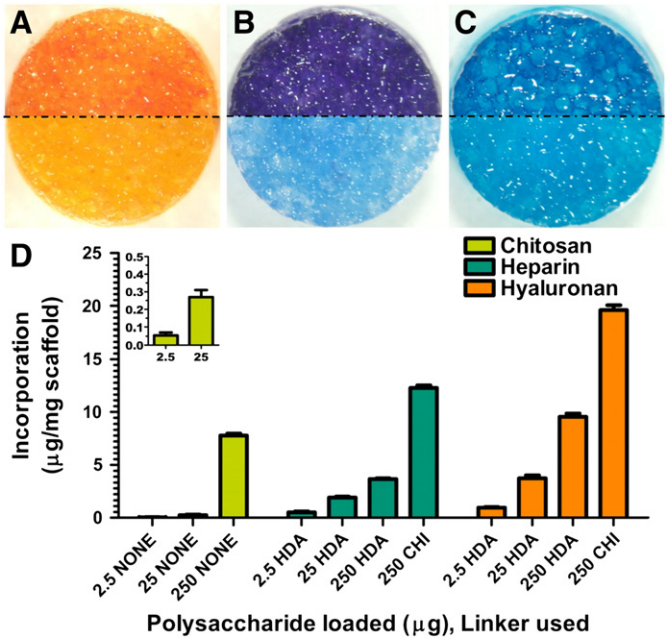

Fig. 1. Polysaccharide conjugation at loading amounts ranging from 2.5 to 250 μg. (A) Orange II association on chitosan-modified (top) and unmodified (bottom) scaffolds. (B) Toluidine blue association with heparin-modified and unmodified scaffolds. Heparin conjugation also resulted in a characteristic metachromasia from sky blue to a deep purple. (C) Alcian blue association with hyaluronan-modified and unmodified scaffolds. (D) Quantification of polysaccharide conjugation by measuring the extinction of absorbance for these dyes at known wavelengths (480 nm, 610 nm, and 620 nm respectively). Either 1.5 mg 1,6-diaminohexane (HDA) or 250 μg chitosan (CHI) was loaded onto PLG scaffolds as crosslinkers for the conjugation of heparin and hyaluronan. Insert magnifies low levels of chitosan conjugation at lower loading amounts.

3. Results

3.1. Surface modification of PLG

The objective of functionalizing the scaffolds with polysaccharides requires the incorporation of functional groups onto the PLG scaffold that can support coupling of the polysaccharides. The immobilization of chitosan to the scaffolds was initially investigated due to the ability to directly couple this polysaccharide onto PLG using EDC/NHS chemistry. Scaffolds with immobilized chitosan (Fig. 1A) had enhanced orange II dye association relative to unmodified scaffolds, resulting in deeper hues. The immobilization of heparin and hyaluronan to the scaffolds was subsequent investigated using 1,6-diaminohexane (HDA) and multi-amine chitosan as crosslinkers. The immobilization of heparin could be visualized by toluidine blue staining that resulted in metachromasia from blue to purple (Fig. 1B). The immobilization of hyaluronan could also be visualized by staining with alcian blue (Fig. 1C) with greater immobilization again resulting in deeper colors.

The conjugation efficiency to PLG scaffolds was subsequently quantified with colorimetric assays that measured the absorbance extinction of these dyes when associated to the polysaccharide (Fig. 1D). Substantial quantities of chitosan bound to the scaffolds when loaded with 250 μg of chitosan, whereas scant levels of functionalization were achieved when lower quantities were loaded. As a result, 250 μg of chitosan was loaded onto scaffolds for the remainder of this study. Using HDA (1.5 mg in 10 μL isopropanol, similar to the literature [11]) as the crosslinker, increasing the amount of heparin loaded onto the scaffold from 2.5 to 250 μg increased binding from 0.52 to 3.67 μg/mg scaffold (Fig. 1D). Similarly, increasing the amount of hyaluronan loaded from 2.5 to 250 μg increased binding from 0.97 to 9.56 μg/mg scaffold. Using 250 μg of chitosan as a crosslinker resulted in surface quantities of heparin exceeding 12 μg/mg scaffold and surface quantities of hyaluronan approaching 20 μg/mg scaffold. The enhanced conjugation of hyaluronan compared to heparin likely reflects lower charge repulsion for hyaluronan due to a lower molecular weight and a lower presence of negatively charged sulfates and carboxyls on hyaluronan [28]. The amount of residual chitosan available for additional complexation was subsequently assessed using Orange II, a dye known to associate with chitosan (Supp. 1) [25]. After conjugating heparin or hyaluronan onto chitosan-modified scaffolds, the amount of chitosan associated to the dye was 16.18% and 42.92% of the initial amount, respectively. Chitosan was chosen to be the crosslinker for the remainder of these studies given its superior ability to immobilize heparin and hyaluronan.

Scanning electron microscopy revealed the influence of the surface reactions on the micro- and nano-architecture of the scaffold. The images revealed the reaction conditions for conjugating polysaccharides did not alter the macroscopic scaffold architecture and structural integrity (Fig. 2A, B). For scaffolds modified with polysaccharides, the textures of the surface demonstrated nodules at high magnification (Fig. 2D–F). The nodules were not observed for unmodified scaffolds (Fig. 2C). For chitosan-modified scaffolds, these small nodules are likely crosslinked, gelled chitosan nanoparticles. In the case of heparin- and hyaluronan-modified scaffolds, the nodules are consistent with the observation of polyelectrolyte complexes formed from the combination of ionic and covalent interactions, which has been reported with other biomaterials modified with these polysaccharides [29–31].

3.2. Lentivirus transduction

The association of lentivirus to the scaffold was investigated for the hyaluronan-, heparin-, and chitosan-modified PLG, with unmodified PLG used as a control. All polysaccharides increased the amount of lentivirus that associated with the scaffold relative to unmodified scaffolds,

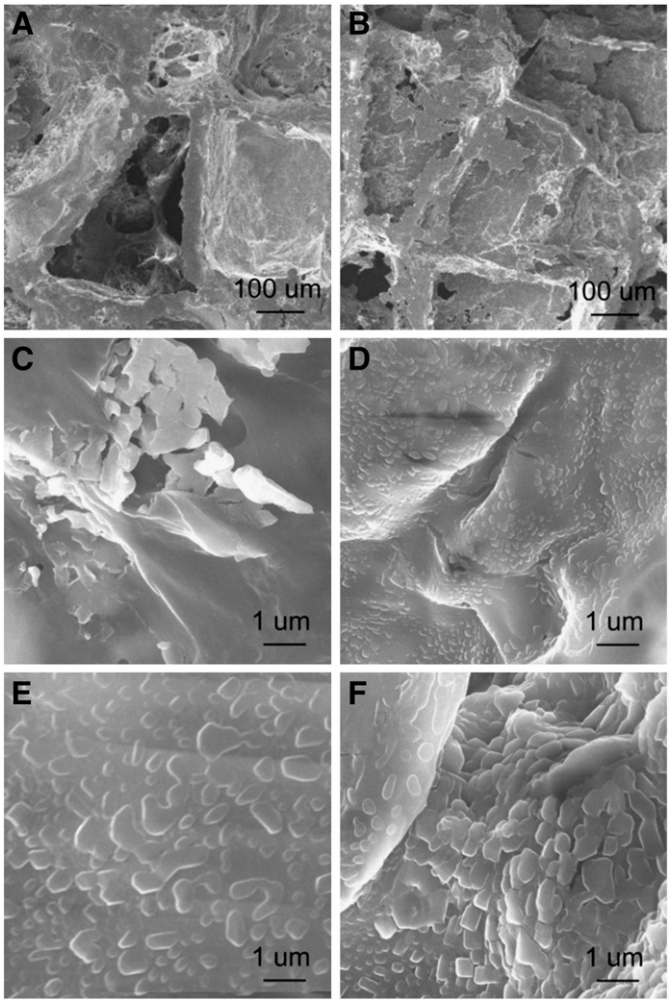

Fig. 2. Scanning electron microscopy of the micro- and nano-architecture of PLG scaffolds. Low-magnification image of (A) an unmodified scaffold and (B) a polysaccharide-modified scaffolds. High magnification image of (C) unmodified, (D) chitosan-, (E) heparin-, and (F) hyaluronan-modified scaffolds.

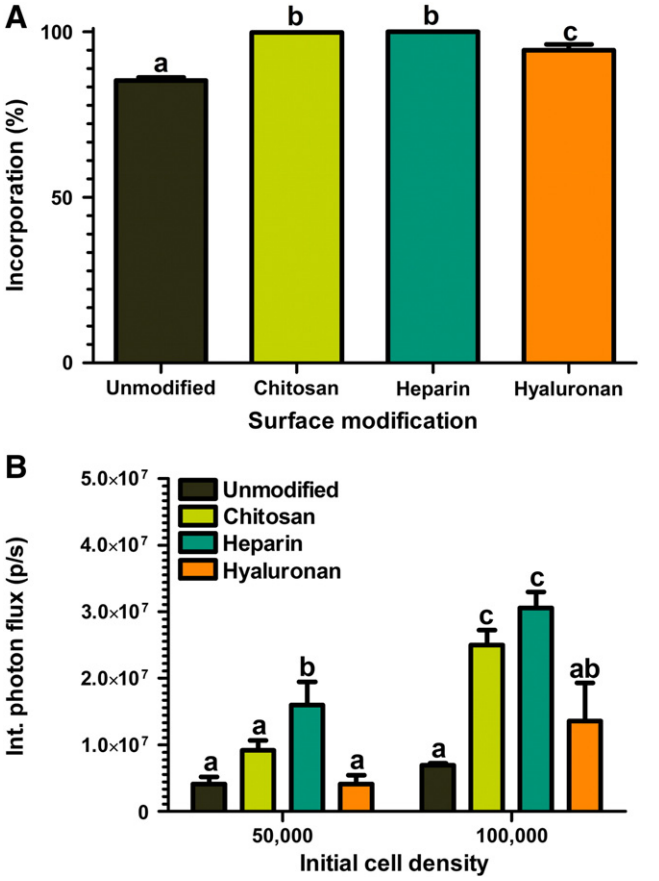

Fig. 3. Lentivirus association to PLG scaffolds at a loading concentration of 4×10^7 luciferase-encoding virus particles. (A) Incorporation of lentivirus onto PLG scaffolds. (B) Gene expression on these scaffolds after 3 days in culture. Conditions designated with different letters are statistically different ($p < 0.05$) using ANOVA with a Bonferonni post-hoc.

with heparin and chitosan having an incorporation efficiency that neared 100% and was significantly greater than the 85% obtained for the unmodified scaffolds (Fig. 3A). Hyaluronan-modified scaffolds had an incorporation efficiency of 95%. The activity of the associated virus was subsequently investigated *in vitro* through cell seeding onto the scaffold at two initial cell densities (Fig. 3B). For all conditions, the greater cell seeding density significantly enhanced expression of the reporter gene luciferase. Relative to unmodified scaffolds, heparin and chitosan provided the greatest fold-increase in transgene expression of 4.4 and 3.6 respectively at the highest seeding density (Fig. 3B). Hyaluronan had expression levels similar to unmodified scaffolds, despite having 10% more lentivirus. Taken together, the heparin and chitosan conditions increased lentivirus incorporation by approximately 15%, yet increased transgene expression almost 4-fold, suggesting that the modification impacted binding as well as virus activity.

Xgal and nuclear fast red staining was performed to determine the cell numbers on the substrates, and whether distinct proliferation rates may explain the differences in transgene expression. The number and distribution of transduced cells within the scaffold were characterized using Xgal staining (blue) to reveal the distribution of cells transduced with Bgal-encoding lentivirus. Heparin- and chitosan-conjugated scaffolds, which had the greatest levels of luciferase production, also had the greatest number of transduced cells, with fewer transduced cells observed on the hyaluronan-modified and unmodified scaffolds. Transduced cells were observed across the scaffold surface (Fig. 4A–D), and were distributed homogeneously throughout the scaffold interior (Fig. 4E–H). The cell number after 3 days of culture was similar in all scaffolds (Fig. 4), as revealed by the number of Xgal and nuclear fast red-stained cells, suggesting differences resulted from transduction efficiency and not cell number.

3.3. Lentivirus activity retention

The retention of lentivirus and virus activity was next investigated as a possible explanation for heparin and chitosan's ability to enhance transduction and transgene expression *in vitro*. For unmodified scaffolds incubated in solution, the lentivirus was released within 48 h (Fig. 5A). Relative to unmodified scaffolds, chitosan slowed the release, with over 40% of the virus retained in the scaffold after 72 h. Interestingly, heparin retained nearly 100% of the lentivirus incorporated onto the PLG scaffold after 72 h. Cells were subsequently seeded on scaffolds that had been incubated for 0 h to 48 h, which would release the loosely associated virus and also decrease the virus activity [3,6]. Lentivirus activity in unmodified scaffolds declined to 5.5% and 3% of its initial expression at 24 h and 48 h respectively (Fig. 5B). Consistent with the initial activity results (Fig. 3B), the chitosan- and heparin-modified scaffolds had a greater activity at the initial time point (Fig. 5B). The activity at 24 h exceeded 70% of the activity obtained at the initial time point. In heparin- and chitosan-modified scaffolds, over two-thirds of their initial activity levels persisted after 24 h, resulting in a 12-fold increase in retained activity relative to unmodified scaffolds. After 48 h, heparin

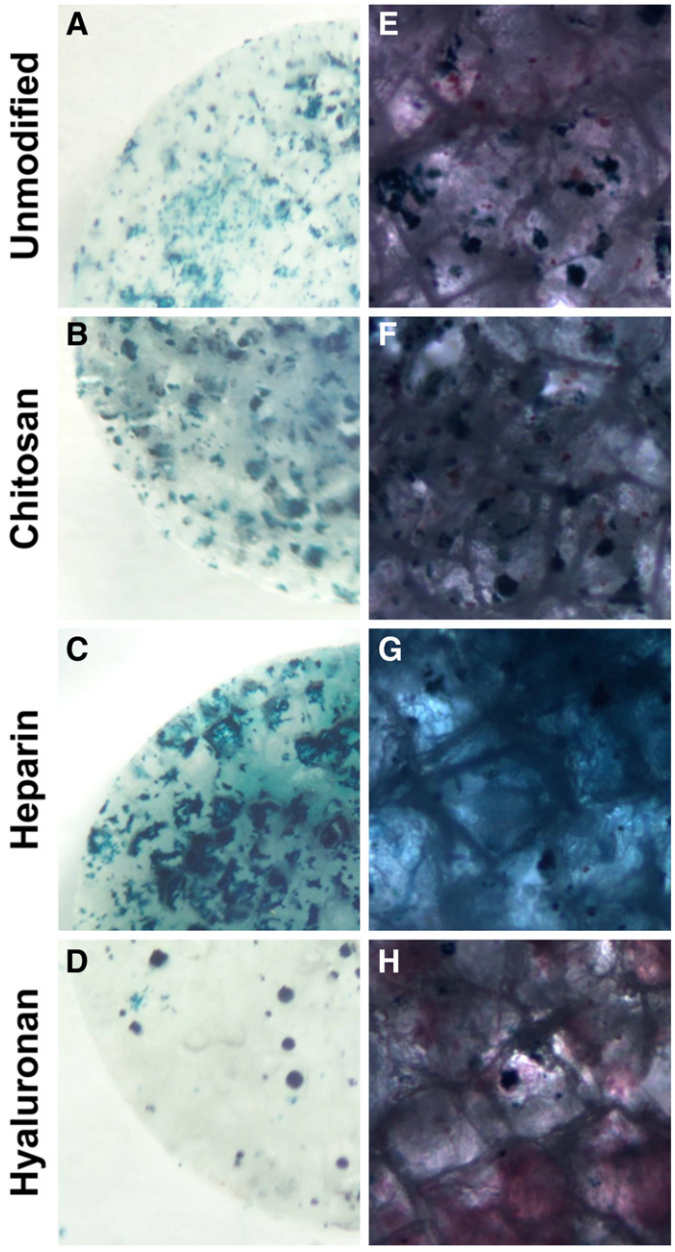

Fig. 4. Gene expression and proliferation on PLG scaffolds loaded with 4×10^7 beta-galactosidase-encoding virus particles. (A–D) Visualization of transduced cells using Xgal on (A, E) unmodified, (B, F) chitosan- (C, G) heparin-, and (D, H) hyaluronan-modified scaffolds after 3 days in culture. Visualization of non-transduced cells with nuclear fast red on (E) unmodified, (F) chitosan-, (G) heparin-, and (H) hyaluronan-modified scaffolds.

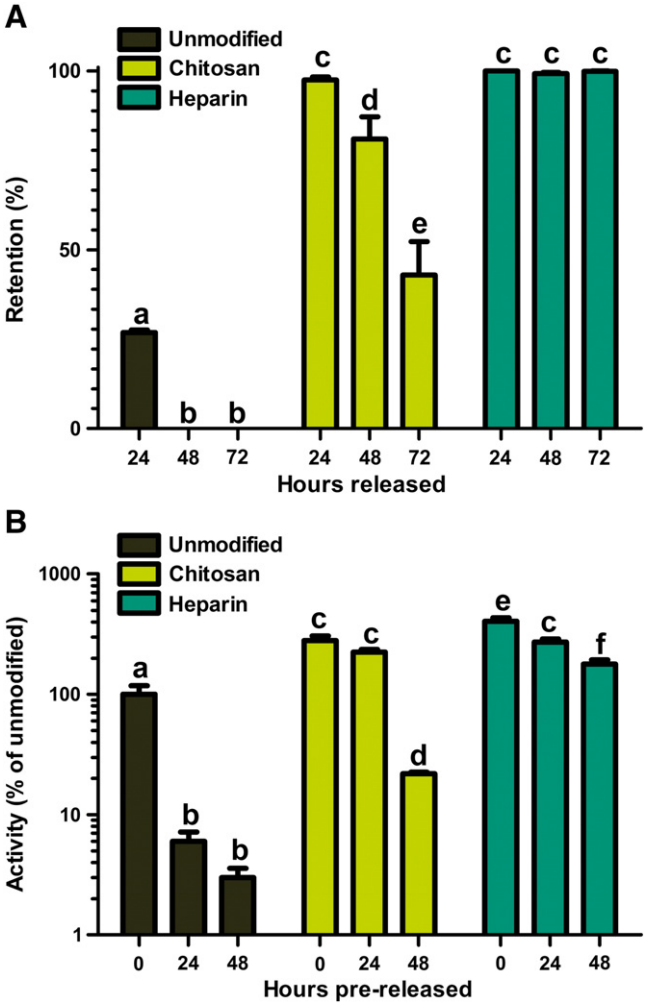

Fig. 5. Lentivirus retention on PLG scaffolds at a loading concentration of 4×10^7 luciferase-encoding virus particles. (A) Release profile of lentivirus on heparin-, chitosan-modified, and unmodified scaffolds 72 h after lentivirus incorporation. (B) Lentivirus activity after pre-releasing lentivirus 0–48 h and culturing for 3 days. Conditions designated with different letters are statistically different ($p < 0.05$) using ANOVA with a Bonferonni post-hoc.

permitted these scaffolds to retain over 40% of its initial expression, and chitosan enabled the retention of nearly 10% of its activity levels. The polysaccharide-coated scaffolds influenced the half-life of the virus, which was determined based on the amount retained on the scaffold and the resulting transgene expression. For unmodified PLG scaffolds, the half-life was 20.8 h, consistent with previous reports of lentivirus in solution [6,32]. With the conjugation of chitosan and heparin to PLG, the lentivirus half-life approximately doubled to 41.5 and 40.4 h respectively. For heparin-modified scaffolds, the decrease in expression with longer pre-release times results from a decline in virus activity; however, for chitosan-modified scaffolds, the decrease in expression levels results from a combination of decrease in virus activity and a decrease in virus amount due to release from the scaffold.

3.4. Porous, multiple channel bridge for mouse spinal cord injury

The next objective was to apply the polysaccharide based immobilization strategy to obtain transgene expression in an *in vivo* model, and we used bridge implantation into a lateral hemisection injury to the mouse spinal cord. Initial studies investigated the attachment of polysaccharides to PLG bridges, which had dimensions of 2.25 mm in length, 1.25 mm in width, and 0.75 mm in height and fit into a hemisection lesion of a mouse spinal cord at the T9–T10 vertebrae (Supp. 2). Bridges were fabricated using a sugar templating process recently developed in our laboratory [21], and had an average channel diameter of 232 ± 5 μm, with a channel cross sectional area exceeding 40%. The addition of salt with the polymer permitted an overall porosity exceeding 90%. The combination of these sacrificial templates using our fabrication technique resulted in an open, interconnected structure that which has previously been shown to promote robust axon growth in a mouse spinal cord injury model [21]. Bridges were modified with heparin or chitosan, and were implanted into a mouse thoracic 10 vertebrae after a hemisection lesion, and compared to unmodified bridges.

The initial cellular response to our bridges was then investigated. Hematoxylin and eosin staining of polysaccharide-modified bridges implanted for 2 weeks revealed that the bridges were well apposed to the contra-lateral tissue and integrated with the host tissue for both polysaccharide-modified and unmodified bridges (Fig. 6A–C). Cells had infiltrated throughout the channels and pores of the bridge, and the channels remained stable for all bridge formulations (Fig. 6D–F). Neurofilament-160 staining displayed the early sprouting of regenerating axons in the middle of the bridge (Supp. 3). Surface modification did not appear to affect the extent in which these had axons grown into the bridge. Neurites were present primarily as individual fibers that were evenly distributed in the channels and pores of the bridge, for all conditions. Double staining for myelin basic protein indicated that some of these neurites were already myelinated and that the extent of myelination was not affected by surface characteristics. Taken together, surface modification of the bridges at this early time-point did not appear to affect the cellular response, which is consistent with the cellular response of bridges implanted without lentivirus in this injury model [21].

3.5. Lentivirus-mediated transgene delivery

We subsequently quantified gene expression from the polysaccharide-modified bridges in the injured spinal cord, where sustained, robust expression has been challenging [2,5]. Luciferase expression was quantified using bioluminescence imaging (Fig. 7), and expression was observed in all conditions for over 8 weeks. Heparin-modified bridges produced the greatest level of expression, followed by chitosan-modified and unmodified bridges. For all bridges, gene expression increased through days 10 or 17 following implantation and then declined at day 24 ($p < 0.05$ for d10 relative to d24 for all bridges), which was consistent with previous reports in the injured spinal cord [2,5] and likely results from the dynamic inflammatory response

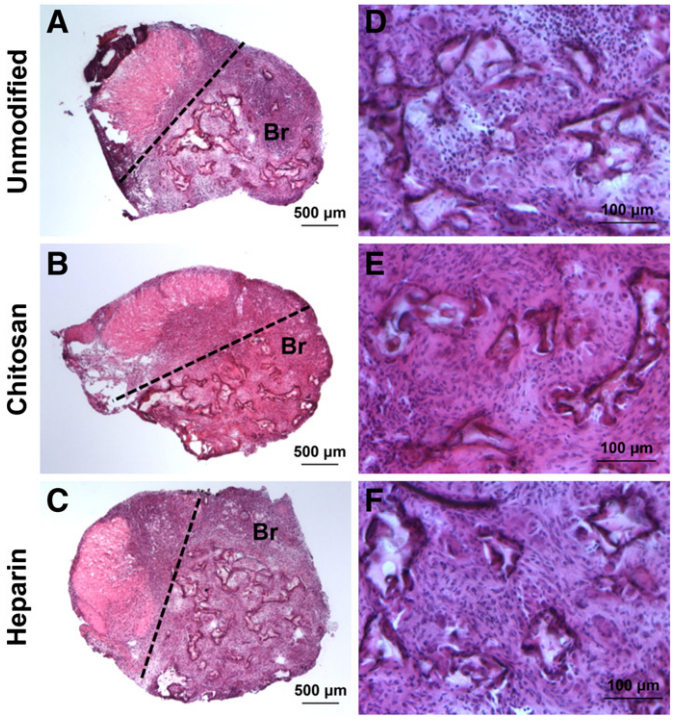

Fig. 6. Hematoxylin and eosin staining in the middle region of the bridge after 2 weeks implantation. Cellular content in (A, D) unmodified, (B, E) chitosan-, and (C, F) heparin-modified bridges. (A–C) Low magnification image of the bridge (denoted by Br) and contralateral side. Dashed line denotes the separation between the injury site and the contralateral side. (D–F) Images of the injury captured at high magnification.

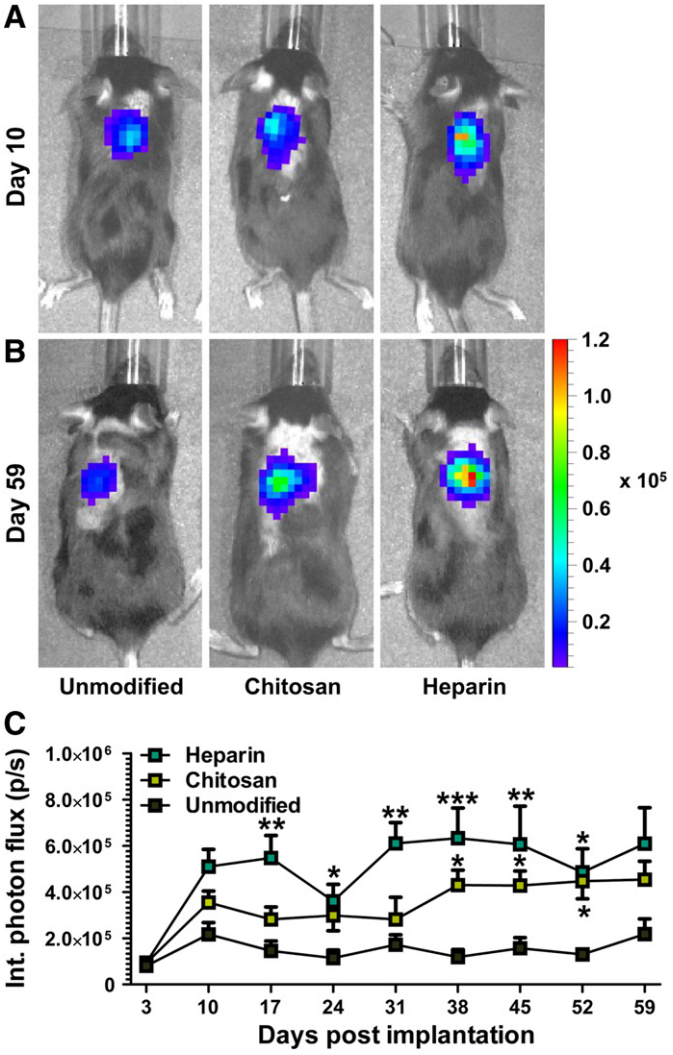

Fig. 7. Gene delivery after spinal cord injury. *In vivo* bioluminescence imaging of transgene expression at day 10 (A) and day 59 (B) *in vivo*. (C) Quantification of light emission at multiple time points. Asterisks indicate statistical significance to unmodified bridges (*: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$) using ANOVA with a Bonferonni post-hoc.

to the injury and biomaterial implantation whereby immune cells enter the material during the acute inflammatory response and exit at later times. Peak expression levels in heparin- and chitosan-modified bridges were 2.5-fold and 1.6-fold greater than that of unmodified bridges respectively. After the initial decline, the expression patterns diverged based on the surface treatment of the bridge. In unmodified bridges, expression steadied at 72% of its peak expression level until the end of the study. In contrast, expression in heparin- and chitosan-modified bridges rose around day 30 and plateaud for the remainder of the study. On average, expression levels after week 5 for heparin- and chitosan-modified bridges were 3.9 fold and 2.7 fold greater than unmodified bridges, respectively.

We characterized the distribution of transgene expressing cells in the injured spinal cord following implantation of lentivirus-loaded bridges. Luciferase-expressing cells (red) appeared in both the bridge and the adjacent host tissue for all conditions (Fig. 8). The cells that stained for luciferase were evenly distributed in the bridges and had distinct morphologies, suggesting multiple cell types were transduced. Heparin-modified bridges appeared to have the greatest number of transduced cells inside the bridge, followed by chitosan-modified bridges and then unmodified bridges. The higher number of luciferase-expressing cells in heparin- and chitosan-modified bridges suggests the

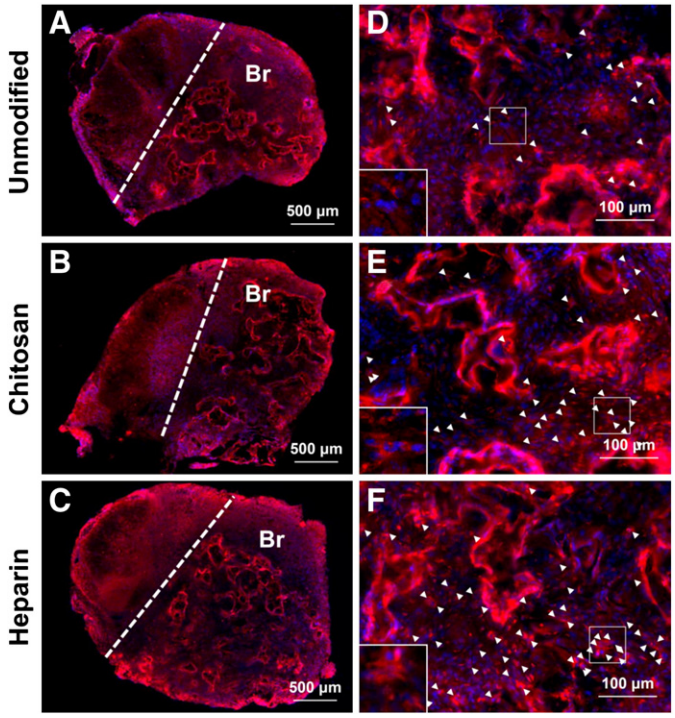

Fig. 8. Transduction after 2 weeks implantation. Luciferase-positive cells (red) and non-transduced cells (blue) in (A, D) unmodified, (B, E) chitosan-, and (C, F) heparin-modified bridges. (A–C) Low magnification image of the bridge (denoted by Br) and contralateral side. Dashed line denotes the separation between the injury site and the contralateral side. (D–F) Image of the injury at high magnification. Arrowheads and inserts indicate transduced cells present in the bridges.

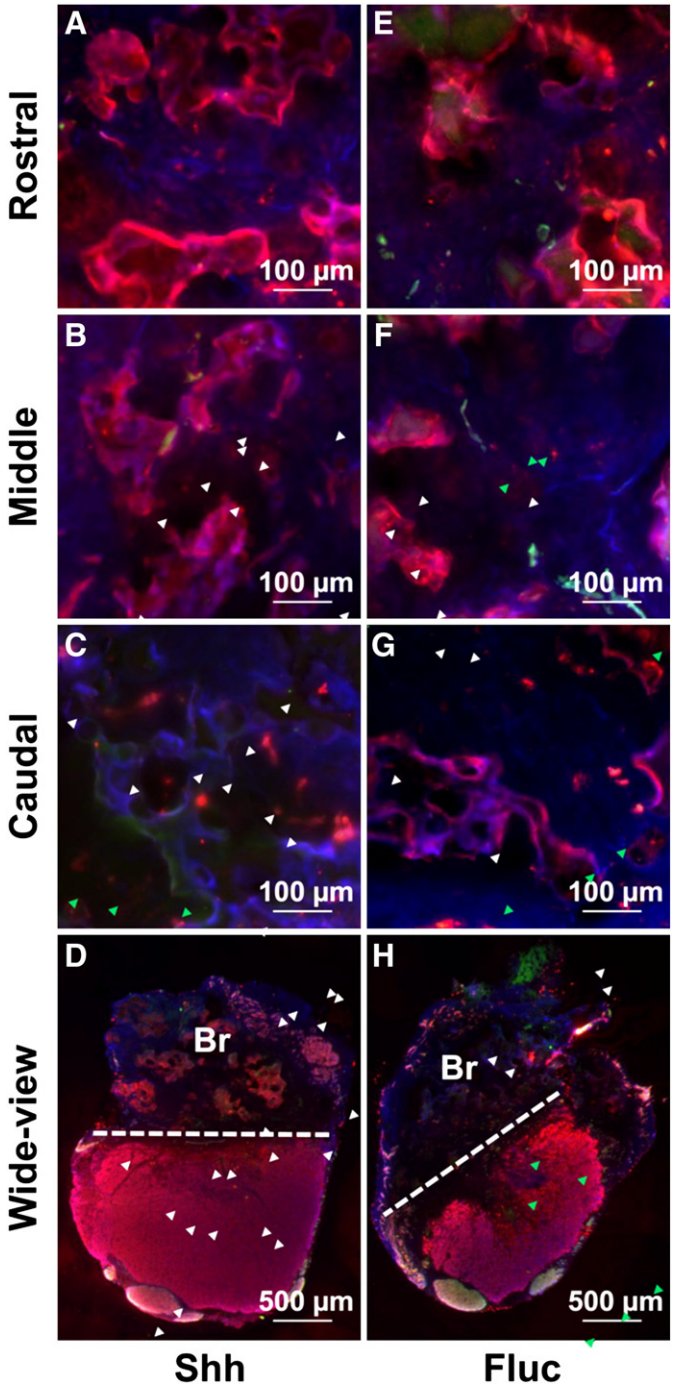

Fig. 9. Axon growth and myelination in heparin-modified bridges after 8 weeks implantation. Neurofilament-160 (blue), myelin basic protein/MBP (red), and p0 (green) staining of bridges that delivered (A–D) Sonic hedgehog (Shh)- or (E–H) luciferase (Fluc)-encoding viruses at (A, E) rostral, (B, F) middle, ad (C, G) caudal sections within the bridge site. Lower magnification images of (D) Shh- and (H) Fluc-delivering bridges (denoted by Br) and contralateral side. Dashed line denotes the separation between the injury site and the contralateral side. White arrowheads indicate oligodendrocyte-derived myelin fibers (MBP+/p0−) present in the bridges. Green arrowheads indicate Schwann cell-derived myelin fibers (p0+) present in the bridges.

increase in expression levels due to chitosan- and heparin-modified bridges was due, in part, to enhanced transduction.

We subsequently investigated the ability of lentivirus-delivering, heparin-modified bridges to promote regeneration after spinal cord injury (Fig. 9). Tissues were retrieved after 8 weeks implantation, and the extent of axon growth (neurofilament/NFM, blue) and myelination (myelin basic protein/MBP, red) throughout the bridge, as well as the source of myelination (p0, green or white) was compared in histological sections for animals that received sonic hedgehog (Fig. 9A–D) or luciferase (Fig. 9E–H) as a control. The induced expression of sonic hedgehog from the biomaterial increased the number of myelinated axons (MBP + or p0+) present in the bridge. The additional myelin tended to be oligodendrocyte-derived (MBP+/p0−, red) and not Schwann cell-derived (p0+, green or white), consistent with sonic hedgehog's known role for promoting the differentiation of progenitors into mature oligodendrocytes [33,34].

4. Discussion

In this manuscript we report the surface modification of PLG with polysaccharides enhanced lentivirus retention on scaffolds *in vitro*, and promoted transgene expression *in vivo*. The advantage of lentivirus as a gene therapy vector is its relative ease of production, broad tropism *in vivo*, and minimal inflammatory response [35]. Previously, we reported incorporation of lentivirus on PLG scaffolds enhanced transduction [4] and maintained the bioactivity of the vector *in vitro* [5]. Here, we demonstrated the utility of lentivirus-immobilized PLG scaffolds *in vivo* in a mouse spinal cord injury model. The mouse model permits great versatility for investigating cellular mechanisms due to availability of transgenic strains. A fabrication technique was recently developed that permitted the fabrication of bridges with a high channel density [21]. However, the small size of the bridges was challenging due to their small size and potential for vector depletion. The minimal interaction between lentivirus and PLG [4] resulted in low transduction efficiency in the injured spinal cord. This challenge motivated the immobilization of polysaccharides to allow for greater retention of the

vectors relative to unmodified scaffolds, while also preserving the virus activity and scaffold stability.

Surface modification of PLG has been a challenge historically, as PLG has a hydrophobic interface and limited number of reactive groups (hydroxyls and carboxylic acids) [36]. Modification of PLG surfaces using EDC/NHS chemistry was achieved by functionalizing the material with multi-amine HDA or chitosan for subsequent coupling of heparin or hyaluronan. The initial strategy for increasing the amine content on the scaffold was to partially hydrolyze the polymer to create more free carboxylic acids that can react with HDA [11,36]. Herein, we applied HDA at its solubility limit to the PLG scaffolds. HDA was most effective at binding polysaccharides at the greatest loading concentration; however, this strategy can compromise the mechanical integrity of the scaffold [11]. Chitosan more effectively immobilized polysaccharides, likely due to an increased number of free amines available for reacting with the other polysaccharides. The effective functionalization of the scaffold combined with maintenance of the structural integrity supports the use of chitosan as a multi-amine crosslinker for future studies.

The use of chitosan to enhance transduction is consistent with the use of positively charged molecules to enhance lentivirus transduction [3,6,37–40]. Chitosan, a cationic polysaccharide [29], supported the binding of lentivirus and prolonged its half-life to promote increased transduction and long term transgene expression *in vivo*. Chitosan's positive charge suggests it enhanced lentivirus transduction using a similar mechanism: shielding the repulsion between the negatively-charged virus coat and the negatively-charged plasma membrane of target cells [41]. A reduction in this repulsion between virus and cells likely enhanced association of the virus to promote internalization [42,43]. In contrast to these reports, heparin—an anionic polysaccharide [29]—also supported lentivirus binding and prolonged half-life. Relative to chitosan, heparin prolonged lentivirus retention and had greater transgene expression *in vitro* and *in vivo*. Heparin has been shown in previous reports to bind to lentivirus and other VSVg-pseudotyped retroviruses [20,44–46]. However, this interaction with lentivirus was not due solely to its negative charge, as scaffolds modified with hyaluronan, another negatively charged polysaccharide [29], did not exhibit enhanced lentivirus incorporation or transduction. Heparin is naturally found on the surfaces of nearly all cell types, and has been shown to facilitate cellular adhesion to biomaterials [47,48]. Thus, the specific binding of heparin to both target cells and lentivirus can lead to greater association of lentivirus to the cell surface, which would promote internalization, and thereby enhance transduction.

The extent of expression using lentivirus *in vivo* was mediated by the combination of virus retention and cell infiltration, consistent with previous reports [7]. Modification of PLG's surface with these polysaccharides promoted greater binding of the virus and thus retention within the material without adversely affecting cell migration into the bridge, as the open architecture of bridges permitted complete cell infiltration by 2 weeks of implantation. Lentivirus-mediated gene expression in the injured spinal cord was bi-phasic. The initial increase and subsequent decrease in expression level corresponded with the dynamics of the foreign body response to the material that occurs after injury. The initial rise in expression may be due to the transduction of transient cell populations, such as macrophages. Previously, we reported that macrophages are transduced by lentivirus delivering bridges [2]. Macrophages have also been reported to increase in presence the first days after injury and then decline in number [26,49,50], which could lead to a decline in expression that is observed between days 17 and 24. An increase in expression was observed after day 24 for the chitosan and heparin-modified bridges, whereas gene expression in the unmodified PLG bridges remained steady at 72% of peak levels. This increase in expression for chitosan and heparin modified bridges is consistent with an increase in transduction of cell types that proliferate and reside in the spinal cord as it regenerates, such as fibroblasts or Schwann cells. Previously, we noted that fibroblasts and Schwann cells are also transduced by lentivirus-delivering bridges [2]. Unlike the inflammation-mediating cells, the presence of these cells in and near our bridges persists for weeks [26].

In conclusion, we investigated lentivirus immobilization to PLG scaffolds with multiple architectures. These scaffolds were modified at the surface using EDC/NHS chemistry to incorporate both positively- and negatively-charged polysaccharides. Heparin and chitosan were shown to enhance the association of lentivirus to PLG, which increased retention within the macro-porous scaffolds. These polysaccharides also extended the half-life of lentivirus to enhance its transduction efficiency *in vitro*. A porous, multiple channel PLG bridge was employed to investigate gene delivery *in vivo* after a lateral hemisection lesion of a mouse spinal cord. Enhanced transduction *in vitro* translated to robust, sustained gene expression in the injured spinal cord. Together, these studies reveal the potential for polysaccharides to promote gene delivery from PLG scaffolds for use in regenerative medicine applications.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.jconrel.2013.06.013.

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