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Publication Date	2011-11-01
Publication Information	McMahon, SS,Nikolskaya, N,Choileain, SN,Hennessy, N,O'Brien, T,Strappe, PM,Gorelov, A,Rochev, Y (2011) 'Thermosensitive hydrogel for prolonged delivery of lentiviral vector expressing neurotrophin-3 in vitro'. Journal Of Gene Medicine, 13 :591-601.
Publisher	Wiley
Link to publisher's version	http://dx.doi.org/10.1002/jgm.1613
Item record	http://hdl.handle.net/10379/4229
DOI	http://dx.doi.org/DOI 10.1002/jgm.1613

Thermosensitive Hydrogel for Prolonged Delivery of Lentiviral Vector Expressing Neurotrophin-3 *in vitro*

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Short title: Lentiviral release from chitosan scaffolds

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Abstract

Background

The development of tissue engineering scaffolds for gene delivery has the potential to enhance gene transfer efficiency and safety via controlled temporal and spatial delivery. Lentiviral delivery can be carried out using the natural biopolymer thermoresponsive gel, chitosan/β-glycerol phosphate (β-GP) as a carrier.

Methods

Three chitosan/β-GP scaffolds were prepared with varying concentrations of chitosan and β-GP to obtain a pH and gelation temperature suitable for in situ delivery. A lentiviral vector expressing either green fluorescent protein (Lenti GFP) or Neurotrophin-3 (Lenti NT-3) was incorporated into the chitosan/β-GP scaffolds and also into collagen 0.1% w/v (control). Viral elution medium was removed at various time points and added to the culture medium of pre-seeded HeLa or primary dorsal root ganglia (DRG) cells respectively. Green fluorescent protein gene expression was quantified using FACS analysis. The effect of lenti.NT-3 was analysed by measuring DRG neurite outgrowth.

Results

Collagen displayed its most significant elution of virus on day one and chitosan/β-GP (with a final concentration of 2.17% chitosan) on day three.

Conclusions

The system shows promise for in-situ, thermoresponsive delivery of lentiviral vectors providing long term gene expression for therapeutic factors to treat conditions such as injury to the nervous system.

Keywords

Introduction

The failed regeneration of axons in the injured central nervous system (CNS) is caused by a number of factors which include the lack of availability of growth promoting substances within the injured area (Becker et al., 2003; Fry, 2001). Lentiviral vectors are well suited for gene delivery to the CNS due to their ability to transduce non-dividing cells and to integrate into the host genome providing long term transgene expression (Azzouz et al., 2004; Blomer et al., 1997; Naldini et al., 1996; Wong et al., 2006). To treat injuries within the CNS, many studies have administered neurotrophic factors to the site of injury (Zhou et al., 2003; Rooney et al., 2009; Taylor et al., 2006). Neurotrophin-3 has been shown to have a regenerative effect on injured spinal cord and to induce axonal plasticity after injury (Hari et al., 2004; Montazeri, and Skutella, 2003). Viral vector delivery of NT-3 in lesioned spinal cord has been described recently using lentivirus (Taylor et al., 2006; Hendriks et al., 2007).

Virus delivery from hydrogels is particularly attractive because the bioactive components readily mix with hydrogel solutions (Cresce et al., 2008; Gustafson et al., 2010; Ishii et al., 2008; Padmashali and Andreadis, 2011; Shin and Shea, 2010). The release kinetics of viral vectors from hydrogel scaffolds are dependent on the physical structure, degradation profile and interaction of gel with the vector (Breen et al., 2006; Chandler et al., 2000). Thermosensitive scaffolds are natural or synthetic materials that demonstrate a solution to gel transition at $37^{o}C$ body temperature. Pluronic gels have recently been used to deliver lentivirus to cells of the CNS (Strappe and Hampton, 2005).

The thermosensitive properties of chitosan have been well documented (Bhattarai et al., 2010; Cheng et al., 2010; Qi et al., 2010). Chitosan is an amino-polysaccharide hydrogel whose properties are largely determined by the degree of deacetylation (DDA; Berger et al., 2004; Ruel-Gariépy et al., 2000). By altering the DDA, the degradation rate can easily be controlled for virus delivery. Due to cationic nature of chitosan and the anionic nature of

most human tissue, chitosan solutions can allow for increased retention of the gel at the injection site. Chitosan is pH sensitive and has been developed for many pH dependent drug delivery systems (Berger et al., 2005). Chitosan has also been shown to seal nerve cell membranes, and thus has a neuroprotective effect following acute spinal cord injury (Cho et al., 2010). Chitosan has also been used as a carrier of NT-3 to promote differentiation of neural stem cells in vitro and to repair of the adult rat hippocampus (Li et al., 2004; Mo et al., 2010). Chitosan scaffolds can be physically or chemically crosslinked. Chitosan/β-GP is a novel physical crosslinked hydrogel that contains ionic, hydrophobic and hydrogen bonds (Chenite et al., 2001). Although chitosan/β-GP-Lentiviral vectors have not been reported previously, smaller molecules such as albumin, have shown retention in chitosan/β-GP scaffolds, via binding of negatively charged ammonium groups (Ruel-Gariépy et al., 2000).

In this study we demonstrated that release of lentiviral vectors from chitosan/β-GP scaffolds is slower than from collagen controls. Lentiviral vectors provide long term gene expression in transduced cells. Delivery of therapeutic genes in the early stages following spinal cord injury may result in apoptosis of the transduced cells. The release mechanism described here provides an ideal delivery system for lentiviral vectors encoding therapeutic genes such as NT-3 in the promotion of axonal regeneration following CNS injury.

Materials and Methods

Collagen Preparation

The preparation of collagen was carried out on ice under sterile conditions. A 0.1% weight / volume (w/v) collagen solution was prepared by mixing DMEM and collagen stock solution. The collagen solution was neutralised using 2M NaOH initially, followed by 0.2M NaOH when a color change was observed. The NaOH was added drop wise and mixed after each

drop. The pH was tested after every drop using pH strips. When the solution reached pH 7, 200μl aliquots were transferred to 1.5ml eppendorfs.

Chitosan and β-GP Preparation

The preparation of chitosan (MW 50,000 – 150,000 g/mol, 70 – 90 % DDA) was carried out on ice under sterile conditions. Several concentrations of β-GP were prepared for neutralisation of chitosan solution for optimal results. All β-GP solutions are sterilised by syringing the solution through a 0.20μm filter. A 5% w/v chitosan stock solution was initially prepared by dissolving chitosan powder in 0.1M HCl solution. Chitosan solution was sterilised using ultra violet light. Chitosan/β-GP scaffolds were prepared by neutralising the acidic chitosan solution with the mildly basic β-GP solution. Chitosan/β-GP scaffolds were prepared using different concentrations and amounts of β-GP to give scaffolds of varying % w/v of chitosan. Three chitosan/β-GP w/v solutions were prepared: Chitosan 3.12 % w/v, β-GP 1.63M, Chitosan 2.38 % w/v, β-GP 1.63M and Chitosan 2.17 % w/v, β-GP 1.08M. The β-GP solution was added drop-wise over a period of 1hr mixing after every drop. The pH was tested after every drop using pH strips as above. When the solution reached pH 7, 200μl aliquots were transferred to 1.5ml eppendorfs.

Scanning Electron Microscope (SEM)

The physical structure of the 0.1% w/v collagen and 2% w/v chitosan/β-GP scaffolds were examined using SEM. Collagen and chitosan/β-GP solutions were prepared as above and at 37°C for 2 hours to allow gelation of the scaffolds. The samples were rinsed with 0.1 M phosphate buffer, pH 7.2. A 2.5% glutaraldehyde fixative was added to the samples until they were completely covered and left at room temperature overnight. The fixative was removed and the samples were rinsed with 0.1M phosphate buffer. The buffer was removed and 50%

ethanol was added. The samples were left to dehydrate at 4ºC for 5 minutes. The alcohol was removed and replaced with fresh 50% ethanol. Again, the samples were left to dehydrate for 5 minutes at 4 ºC. This step was repeated using 75%, 80%, 90% and 100% ethanol. The alcohol was removed and the dehydrated samples were transferred to glass vials. Hexamethyldidilazane was added to the glass vials until the samples were completely covered and left at room temperature for 30 minutes. The samples were left at room temperature to air dry. Each sample was then mounted on an SEM carbon-painted stub and gold-coated with a sputter-coater. Samples were visualized with a Hitachi S-4700 SEM using an accelerating voltage of 10kV.

Fourier Transform Infrared Spectroscopy (FTIR)

Fourier Transform Infrared Spectroscopy was used to identify chemical compounds and substitute groups within chitosan powder, β-GP powder and chitosan/β-GP [2.38% w/v, 1.64M] and chitosan/β-GP [2.17% w/v, 1.08M] scaffolds. Chitosan/β-GP solutions were prepared as described above. The solutions were aliquoted at 200µl to 1.5 ml eppendorfs for 2 hours at 37°C. FTIR analysis was carried out using a Shimadzu 8300 FTIR.

Lentiviral vector production

The human NT-3 sequence was cloned from pSK-NT-3 plasmid (a kind gift of John Rogers and James Fawcett) by Ecor1 digestion and ligated into the pGEM 7Z(+) plasmid (Promega). The sequence was then excised by BamH1 and Xho1 digestion and ligated into the lentiviral vector cPPT-GFP replacing the GFP sequence. cPPT-GFP lentiviral vector is a derivative of the parental HR'GFF lentiviral vector (a kind gift of Didier Trono) containing the HIV-1 cPPT sequence cloned into the unique Hpa site (15). 293T cells were cultured to 70% confluency in 10cm dishes in DMEM (Gibco) containing 10% FCS (Sigma), 1% L-glutamine

(Sigma) and 1% pen/strep (Sigma). Lentiviral vector was produced by co-transfection of 293T cells with Lenti-NT-3 or Lenti GFP using a calcium phosphate transfection kit (Invitrogen). Medium was replaced 24 hours post transfection and supernatant containing viral vector was collected at 48 and 72 hours post transfection. Medium was filtered through a 0.45µm filter and centrifuged for 2.5 hours at 25000rpm and 4^0C using a Sorvall surespin 630 swing out rotor. The pellets were resuspended in 50µl PBS containing 1% BSA and left overnight at 4^0C to resuspend viral vector. Aliquots of lenti NT-3 and lenti GFP were stored at -80^oC.

Lenti GFP titration

Titration of Lenti GFP viral stocks was carried out in order to consistently use the same titre virus in elution from scaffolds experiments. Hela cells (grown in same media type as shown for 293T cells above) were seeded at a density of 1 x 10^5 cells per well in 6 well trays. One day later, lenti-GFP was added to the 6 well trays in triplicate for each serial dilution. 72 hours post transduction HeLa cells were trypsinised and fixed with 4% paraformaldehyde, washed with PBS and analysed by FACS analysis of GFP expression using Guava cytosoft instrument and 'ExpressPlus' software (Guava Technologies, Philadelphia, USA). The dilution resulting in approximately 20% GFP-positive cells was used to calculate transducing units (TU)/ml.

Lenti NT-3 titration

The amount of NT-3 produced from cells was measured following lenti NT-3 transduction of Hela cells and ELISA analysis. Hela cells were plated at 1 x 10^5 cells per well in 6 well dishes. 10µl and 5µl of concentrated Lenti NT-3 was added to cells in triplicate and medium was replaced 24hours following transduction. At 72 hours post transduction the medium was

tested for NT-3 using an NT-3 ELISA immunoassay kit (Promega). Each sample of cell culture medium was serially diluted and tested in triplicate.

Elution of Lenti GFP from scaffolds

Solutions of 0.1% w/v collagen and chitosan 3.12 % w/v, β-GP 1.63M, chitosan 2.38 % w/v, β-GP 1.63M and chitosan 2.17 % w/v, β-GP 1.08M were prepared in 1.5ml eppendorfs as described above. Lenti GFP or lenti NT-3 was added to collagen or chitosan/β-GP scaffold solutions at a volume of 8µl virus : 200µl scaffold solution. The virus and scaffold solution was triturated up and down several times using a Pasteur pipette to ensure even mixing and incubated at 37°C for 2 hours to allow gelation. 200µl Hela medium (viral elution medium) was then added to the surface of the gel scaffold. Samples were prepared in triplicate for collagen and chitosan gels. Three elution time points were examined for each collagen and chitosan gel at 1 day, 2 days and 3 days. At each elution timepoint medium was removed from the gels. Three scaffolds with no virus negative controls (control elution media) were also prepared for each time point.

To analyse the elution profile of the lentiviral vector from the scaffolds, 100µl elution medium was removed from gelated scaffolds. The elution medium from Lenti GFP containing scaffolds was added to HeLa cells preseeded at a density of 1×10^5 cells/well in 24 well trays. Hela cells were grown for 3 days in triplicate in either control medium or viral elution medium. Elution of lenti GFP and expression of GFP in Hela cells was examined by FACS analysis of GFP expression using Guava cytosoft instrument and 'ExpressPlus' software to determine the percentage of GFP expressing cells.

Elution of Lenti NT-3 from scaffolds

Solutions of 0.1% w/v collagen and chitosan 2.17 % w/v, β-GP 1.08M were prepared in 1.5ml eppendorf as described above. Lenti NT-3 was added to collagen or chitosan/β-GP scaffold solutions at a volume of 8μl virus : 200μl scaffold solution. The virus and scaffold solution was triturated to ensure even mixing and incubated at 37°C for 2 hours to allow gelation. 200μl DRG medium (viral elution media) was then added to the surface of the gel scaffold. Samples were prepared in triplicate and there were four elution time points; 1 day, 2 days, 3 days and 7 days at which elution medium was removed. Three scaffolds with no virus negative controls (control elution media) were also prepared for each time point. Elution medium (100μl) from Lenti NT-3 scaffolds was removed from gelated scaffolds containing Lenti NT-3 and this medium was added to DRG neurons preseeded at a density of 1×10^5 cells/coverslip (see below for DRG isolation). DRGs were grown for 3 days in triplicate in either control medium or viral elution medium. Elution of lenti NT-3 and its effect on DRGs was examined by immunocytochemical staining of DRGs and analysis of neurite outgrowth using Image J software.

Primary DRG culture

DRGs were dissected from embryonic day 14 – 15 Sprague Dawley rat embryos using a dissecting microscope. Pregnant rats were sacrificed with an overdose of the anaesthetic Euthanol and embryos were dissected out and kept on ice in Hanks balanced salt solution (HBSS, Sigma). DRGs were dissected from the embryos using fine forceps and collected in HBSS. DRGs were spun and resuspended in 0.25% trypsin for 20 minutes at 37°C. To remove trypsin, DRGs were spun and resuspended in DRG medium i.e. MEM (Gibco) containing 20% FCS (Sigma), 1% L-glutamine (Sigma), 0.6% glucose (Sigma), 10ng/ml NGF (R&D Systems), 2×10^{-5}M FudR (Sigma), 2×10^{-5}M Uridine (Sigma). Cells were

triturated to create a single cells suspension. DRG cells were seeded onto 1µg/ml collagen (Sigma) coated coverslips at a density of 1 x 10^5 cells/ coverslip.

Immunocytochemistry

DRG cells were washed three times with PBS following fixation with 4% paraformaldehyde. Cells were incubated in PBS containing 3% normal goat serum (Sigma) block solution for 10 minutes. Cells were incubated with mouse anti-β-tubulin primary antibody (Chemicon) diluted 1:100 in block solution for 1 hour. Cells were washed three times with PBS and incubated with anti-mouse IgG FITC (Sigma) diluted 1:100 in PBS. Secondary antibody was washed off three times in PBS. Coverslips were mounted on slides using Dako antifade mounting medium (DakoCytomation).

Image analysis and Statistics

DRGs were visualized on an Olympus IX81 fluorescent microscope. Three images were captured per coverslip using Volocity software. Image J software was used to analyse the images by converting the image to an 8-bit image and measuring the percentage area covered by DRG neurites in control versus DRGs grown in Lenti NT-3 elution medium.

All data was tabulated and graphed using Microsoft Excel. Statistical calculations were performed using Minitab software. A one-way analysis of variance was performed to examine effect of elution time and elution media. Post-hoc comparisons were undertaken by a Tukey's test. Differences were considered to be statistically significant at a probability value (P) < 0.05.

Results

SEM analysis of scaffolds

The physical characteristics of collagen and chitosan/β-GP scaffolds were observed using SEM. A 2% w/v chitosan/β-GP scaffold (figure 1A) and a 0.1% w/v collagen scaffold (figure 1B) show very different physical characteristics. Collagen is a relatively porous material whereas chitosan/β-GP is a denser hydrogel with smaller pore size than collagen. It has a very fibrous microstructure with large interconnecting areas between fibres.

FTIR analysis of scaffolds

FTIR examination provides information on the changes in chemical structure following the addition of β-GP. FTIR analysis was carried out on four different samples: chitosan dry powder, β-GP dry powder, chitosan/β-GP [2.17% w/v, 1.08M] and chitosan/β-GP [2.38% w/v, 1.64M].

The transmittance spectrum of dry chitosan powder (Figure 2A) displays numerous bands. The peak at 1515 cm-1 and 1620 cm-1 are attributed to the amine I band. FTIR transmittance spectrum of dry β-GP powder is shown in Figure 2B. The broad band present at 3174cm-1 is representative of both C-H and O-H stretching. The narrow peak present at 1053cm-1 is characteristic of C-O bonds and the sharp peak at 960cm-1 represents P-O bonds.

FTIR transmittance spectra of chitosan/β-GP [2.17% w/v, 1.08M] and chitosan/β-GP [2.38% w/v, 1.64M] in Figures 2C and 2D respectively display some of the same characteristics in both spectra. Transmittance at 1640cm-1 becomes more pronounced in the chitosan/β GP sample with greater chitosan and β-GP concentrations. The base of the peak at 3000cm-1-3500cm-1 is broader in the second chitosan/β-GP sample.

Titering of Lentiviral vectors

Lenti GFP was titered using HeLa cells to determine the transduction efficiency of the batch of virus used in the viral elution experiments. The presence of green fluorescence in HeLa cells was analysed using flow cytometry. Guava flow cytometry results showed that 0.05µl lenti GFP gave a transduction rate closest to 20% (Figure 3A). Using this information, the titre was calculated at 4.7×10^8 TU/ml.

Neurotrophin-3 levels were measured in medium from lenti NT-3 transduced HeLa cells using an ELISA. Levels of between 112 and 168ng NT-3/ml were detected following transduction of HeLa cells with 5 and 10µl lenti NT-3 respectively (Figure 3B).

Lenti GFP elution from chitosan and collagen scaffolds

Elution of Lenti GFP virus from scaffolds and subsequent transduction of HeLa cells by the eluted virus was analysed using flow cytometry.

The chitosan/β-GP system with a final concentration of 3.12% chitosan was set up to examine the elution of lentiviral particles from a scaffold with a high concentration of chitosan. Green fluorescence was observed in the control samples, especially on day 3, which was unexpected as there was no lenti GFP virus present (Figure 4A). The high concentration of chitosan/β-GP may have caused HeLa cells to autofluoresce.

The 2.38 % concentration chitosan allowed the effect of lower chitosan concentration on lentiviral elution to be examined. The results differ significantly from those with 3.12% chitosan. Lenti GFP appears to elute over a longer period of time when incorporated into chitosan scaffolds with a low chitosan concentration. As can be seen from figure 4B, lenti GFP is slowly released at day one with an increased release profile for day two, this then remains constant for day three. However cells incubated with control elution medium again showed a significant increase in green fluorescence in control samples by day three. This may suggest that longer exposure times to chitosan/β-GP increases the scaffolds toxicity. If

toxicity is increased in the in vitro environment, this can result in death of the HeLa cells, which in turn will autofluoresce (see Supplementary Figure 1).

The 2.17% chitosan provided additional information on the effect of lowering chitosan concentration on lentiviral elution. The HeLa cells showed highest GFP expression at day three time point (Figure 4C). The elution of virus from within the scaffold takes slightly longer corresponding with the increased elution seen at day three. The trend of increased autofluorescence observed in control samples as the length of incubation time with the scaffold increases was also observed here.

The collagen scaffold was set up as a control system. Figure 4D shows there seems to be an initial burst release of lenti GFP on day1 followed by a reduction in release on day2 and day3 (see Supplementary Figure 2).

Comparative analysis of virus elution from collagen and chitosan scaffolds on days 1, 2 and 3 is shown in figure 4E. Significant differences in viral elution were observed between the two scaffolds. In comparison to collagen, chitosan seems to provide more controlled and slower elution of Lentivirus from its scaffold, with a concentration around 2% providing a more controlled sustained delivery system for lentiviral elution compared to collagen. Collagen provided an elution profile similar to 3.12% and 2.38% chitosan at the 1 day time point but by day 2 and 3 collagen showed significantly less elution of virus. Chitosan/β-GP with a final chitosan concentration of 2.17% showed the most promising results for elution of virus as this scaffold provided the slowest release compared to the chitosan scaffolds with higher chitosan concentrations.

Lenti NT-3 elution from chitosan and collagen scaffolds

Primary DRG neurons were used in this study as they provide a more true reflection of the response of neurons in vivo. Dorsal root ganglia neurons that are grown in lenti NT-3

containing elution medium will become transduced with lenti NT-3 and hence will start to secrete NT-3 protein, which will in turn promote neurite outgrowth. Comparison of the percentage area covered by DRG neurites showed which elution medium environments provided the best NT-3 secretion. Only the 2.17% chitosan scaffold was used in this elution study as a comparison to collagen, as 2.17% chitosan provided the best elution profile of lenti GFP. A further elution timepoint of 7 days was included to compare prolonged elution from the scaffolds.

The 1 and 2 day timepoints do not show any difference in neurite growth but by day 3 there is a significant increase, which continues up to day 7 since more NT-3 will be secreted as time progresses (Figure 5A). Neurite outgrowth was significantly greater in the DRGs incubated with lenti NT-3 (Figure 5C, E, G, I) elution medium compared to control elution media (Figure 5B, D, F, H).

The four timepoints did not show any difference in neurite growth (Figure 6A). Neurite outgrowth was significantly greater in the DRGs incubated with lenti NT-3 (Figure 6C, E, G, I) elution medium compared to control elution media (Figure 6B, D, F, H).

When both scaffolds are compared, a significantly larger increase in neurite outgrowth was observed in DRGs grown in viral elution medium from chitosan compared to collagen, indicating a greater capacity for elution from the chitosan scaffold.

Discussion

Delivery of therapeutic genes via biomaterial scaffolds and viral vectors has proved an attractive approach to repair of the injured spinal cord in recent years (De Laporte et al., 2009; De Laporte et al., 2010; Samadikuchaksaraei, 2007; Strappe and Hampton, 2005; Lai, and Brady, 2002). A recent study has shown that lentiviral vectors can promote DRG neurite outgrowth in an in vitro model of spinal cord injury (Donnelly et al., 2010).

Although it is not possible to detect any difference in the physical structures of the chitosan/β-GP scaffolds; nonetheless, it is easy to identify fibrous structures in the collagen sample and a more compact chitosan/β-GP structure (Figure 1). Structural changes of chitosan after the addition of β-GP were confirmed by FTIR analysis. The transmittance spectrum of dry chitosan powder (Figure 2A) displays a band at 1515cm-1 and has a larger intensity than at 1620cm-1 which suggests effective deacetylation of the chitin to chitosan (Paulino et al., 2006). The band observed in-between 3000-3500 cm-1 corresponds to the vibrational stretching of the hydroxyl group and extension vibration of N-H and intramolecular hydrogen bonds (Xie et al., 2001). Transmittance at 1640cm-1 becomes more pronounced in the chitosan/β GP sample with greater chitosan and β-GP concentrations. It is possible to observe changes in spectra due to the mere presence of increasing amounts of β-GP functional groups but also changes caused by hydrophobic and hydrogen bonding.

Chitosan elution of lentivirus occurred slowly over time. The elution profile of lentivirus from chitosan is dependent on chitosan concentration, as the concentration of chitosan is increased elution is slowed down, this is obvious even on day 1 where 2.17% chitosan gave the highest elution of virus compared to higher concentrations of chitosan (Figure 4E).. The mechanism of virus release is related to concentration and electrical charge. Chitosan is positively charged and lentiviral particles are negatively charged. If the chitosan is not significantly neutralized using β-GP, the viral particles can become trapped and unable to elute from the scaffold. However, the elution of lentivirus cannot be inhibited by concentrated scaffold alone as the pore size is considerably larger than that of the released virus (100-120nm; Woodley et al., 2004). The difference in chitosan concentrations is insignificant relative to the porosity of the scaffold.

A high level of autofluorescence was observed in control samples in the chitosan/β-GP of higher concentrations. Higher concentrations of chitosan/β-GP scaffolds degrade

faster (Ahmadi and De Bruijn, 2008) resulting in greater levels of fragments present in the elution medium (Molinaro et al., 2002). Toxicity within the in vitro environment may result following increased degradation of the scaffold. If toxicity is increased this can result in death of the HeLa cells, which in turn will autofluoresce. This may be a possible explanation for the high levels of autofluorescence present in HeLa cells within control samples. High concentrations of chitosan/β-GP would be avoided on continuation of this study in an in vivo model.

It is our hypothesis that viral elution is inhibited in the chitosan/β-GP scaffold due to an increased charged density and bonding within the polymer network (Arsianti et al., 2010; Ghosh et al., 2008; Zou et al., 2009). In chitosan/β-GP scaffolds, with a neutral pH, approximately 17% of amine groups are still protonated (Chenite et al., 2000). An increase in hydrophobic ($-CH_3$) and hydrogen bonding favoring groups (-OH, -NH and C = O) allows for a more compact gel formation due to increased intermolecular bonding, entrapping lentivirus within the scaffold. The most significant elution of lenti GFP from collagen scaffold was seen on day one. Collagen hydrogels have a very small concentration of collagen (0.1% w/v) therefore are very porous, producing a delivery system which provides very fast elution. Collagen is a well defined 3-D scaffold previously used in viral delivery. Transduction of HeLa cells is highest on day 1 suggesting a burst release of lentivirus in the first 24 hours. A temporal controlled release of virus is essential to increase the transduction efficiency of the viral vector (Dreesen et al., 2009; Orlando et al., 2010; Strappe et al., 2005). In addition to release rate, other factors are important to consider in vivo including pseudotyping with distinct viral envelopes and sustained high-level transgene expression (Kauss et al., 2010; Mátrai et al., 2010). Although a sustained release of macromolecules over a period of several hours to a few days has already been established in chitosan/β-GP (Ruel-Gariépy et al., 2000), lentivirus release kinetics from chitosan/β-GP has not been studied previously.

Transduction of DRGs with elution medium containing lenti NT-3 provided a source of NT-3 protein within the DRG in vitro environment that promoted DRG neurite outgrowth. This data provides functional significance for the elution of lentiviral vectors from the scaffolds within this study. In the chitosan scaffold, a significant increase in neurite outgrowth was observed at day 3 and this increase continued to day 7, which would be expected as the DRGs are continually secreting NT-3 once they have been successfully transduced. These results are similar to lenti GFP elution, where most elution of lenti GFP from 2.17% chitosan was observed on day 3. Collagen scaffolds eluting lenti NT-3 did not show and difference between day 1 and day 7, although there was a trend toward increase in neurite outgrowth as time in vitro progressed. This is likely because the collagen scaffolds released the virus on day 1, as observed with lenti GFP elution. The controlled release of neurotrophic factors from hydrogels in spinal cord lesioned environments has demonstrated the promotion of neurite fiber sprouting (Burdick et al., 2006; Jain et al., 2006; Piantino et al., 2006; Taylor et al., 2004). The present study provides a more long term approach in that use of lenti NT-3 will provide long term gene expression when eluted at the site of a spinal cord lesion.

The data in this paper provides a basis for a temporally controlled delivery of therapeutic genes, from chitosan/β-GP-lentivirus scaffolds. Currently, viral vectors remain the most efficient means of gene delivery and lentiviral vectors offer long term and stable gene expression (Abdellatif et al., 2006; Wong et al., 2006). The physical and chemical properties of chitosan/β-GP are versatile and can be tuned for optimal lentiviral release.

Acknowledgements

Conflict of interest statement

The authors have no conflict of interest to declare.

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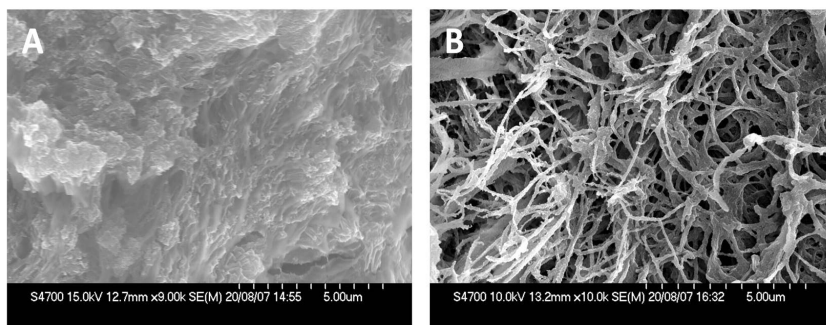
Figure 1

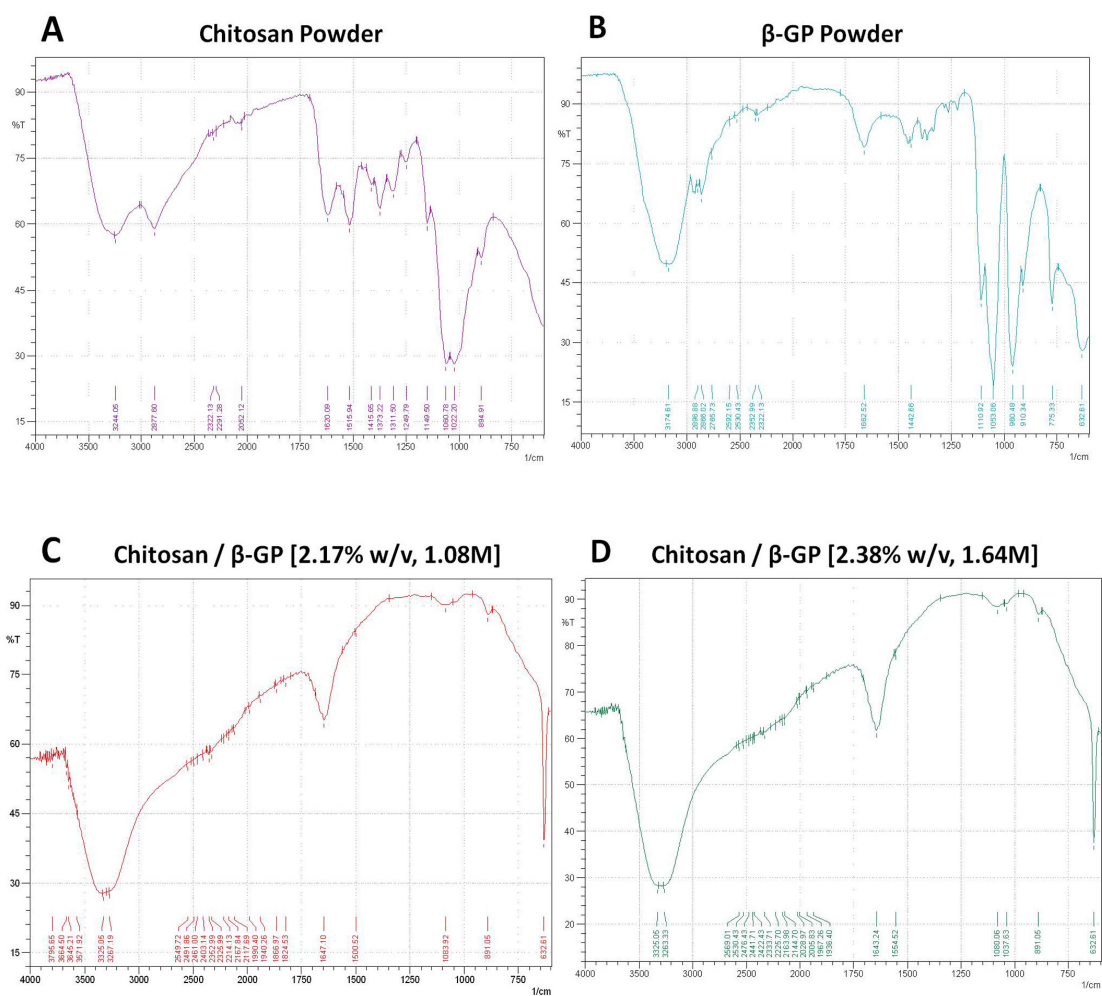SEM images of A: 2% w/v chitosan/β-GP and B: 0.1% w/v collagen. 9000X magnification.

Figure 2

FTIR transmittance spectra of A: chitosan dry powder, B: β-GP dry powder, C: chitosan/β-GP [2.17% w/v, 1.08M] and D: chitosan/β-GP [2.38% w/v, 1.64M].

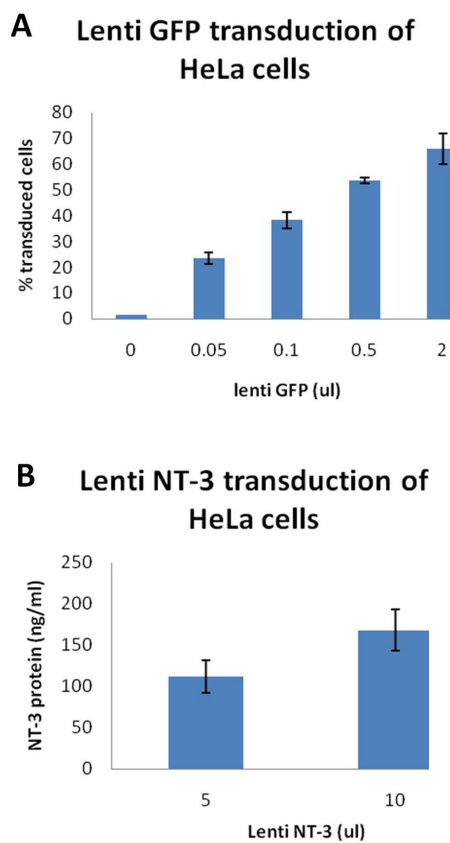**A**   **Lenti GFP transduction of HeLa cells**

B   **Lenti NT-3 transduction of HeLa cells**

Figure 3

Graphs show percentage Lenti GFP transduction of HeLa cells (A) and ELISA data showing

protein concentration produced following transduction of HeLa cells with Lenti NT-3 (B).
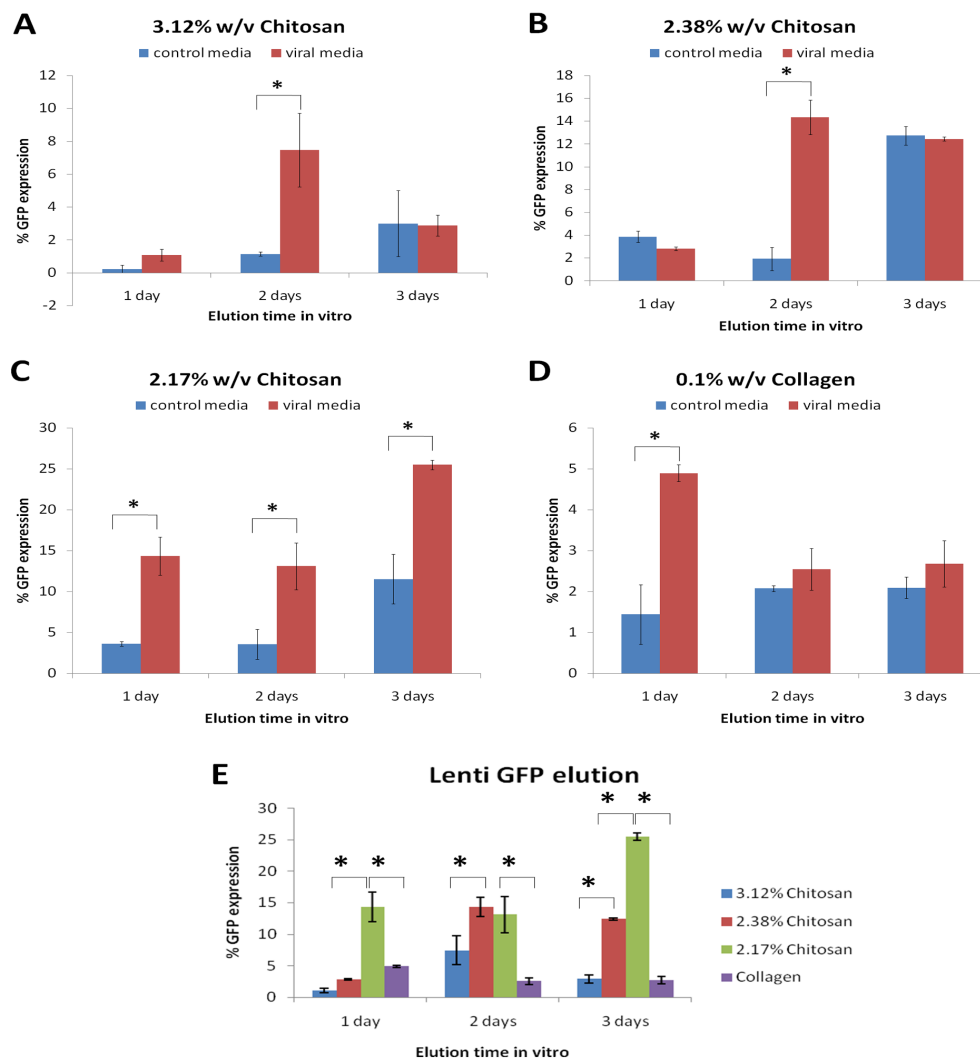
Error bars represent standard deviation

Figure 4

Graphs show percentage of Hela cells expressing GFP after 3 days exposure to elution medium from control and Lenti GFP eluting scaffolds. Scaffolds shown are A: Chitosan 3.12 % w/v, β-GP 1.63M; B: Chitosan 2.38 % w/v, β-GP 1.63M; C: Chitosan 2.17 % w/v, β-GP 1.08M and D: Collagen 0.1% w/v. E shows combined Lenti GFP elution profile from all four scaffolds at the three elution timepoints. Error bars represent standard error of the mean. * = $P < 0.05$.
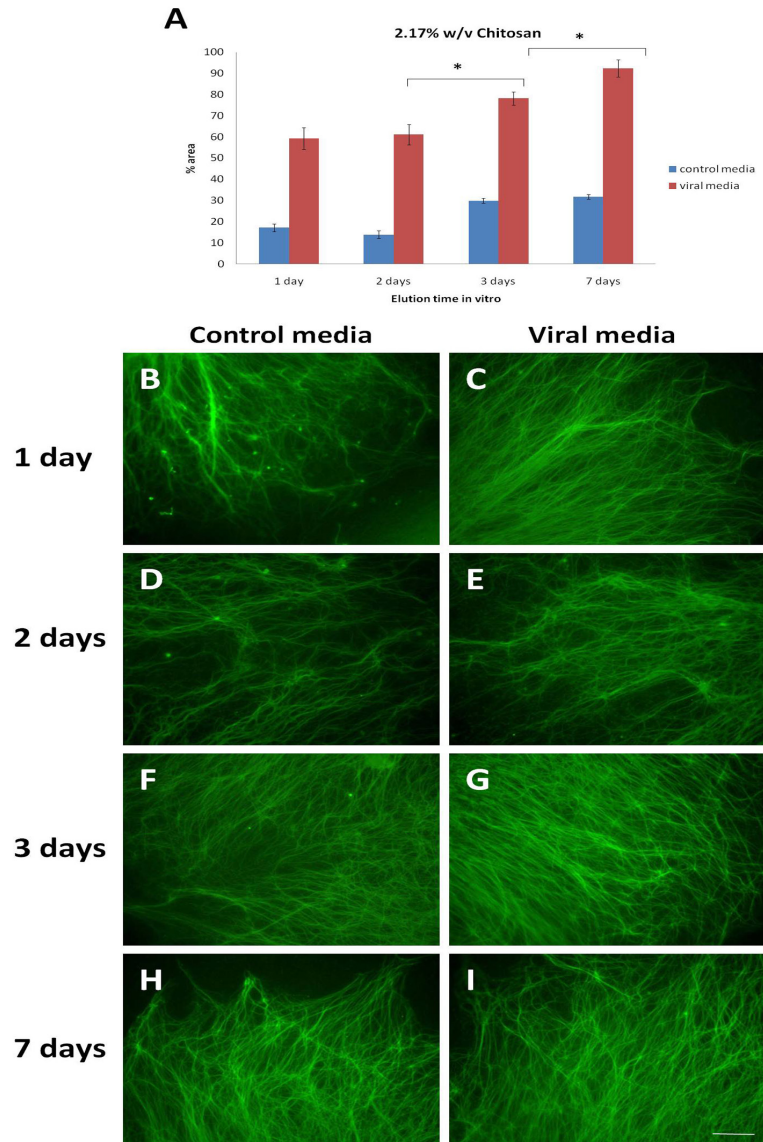
Figure 5

Graph shows percentage area covered by DRG neurites after 3 days exposure to elution medium from control and Lenti NT-3 eluting chitosan 2.17 % w/v, β-GP 1.08M scaffolds (A). Error bars represent standard error of the mean. * = P < 0.05. Fluorescent images show β-tubulin stained DRGs after 3 days growth in elution medium from control (B, D, F, H) and Lenti NT-3 eluting (C, E, G, I) chitosan 2.17 % w/v scaffolds at 1 day, 2 days, 3 days and 7 days timepoints respectively. Scalebar = 100μm.
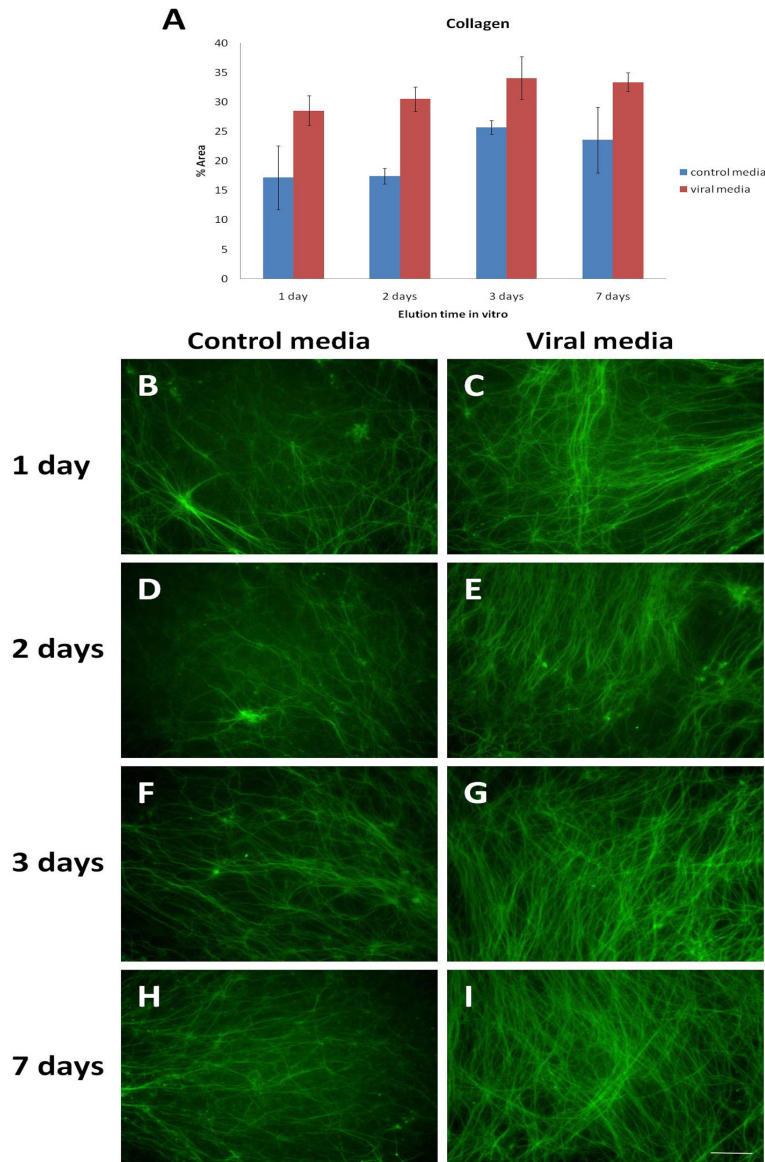
Figure 6

Graph shows percentage area covered by DRG neurites after 3 days exposure to elution medium from control and Lenti NT-3 eluting collagen 0.1 % w/v scaffolds (A). Error bars represent standard error of the mean. * = P < 0.05. Fluorescent images show β-tubulin stained DRGs after 3 days growth in elution medium from control (B, D, F, H) and Lenti NT-3 eluting (C, E, G, I) collagen 0.1 % w/v scaffolds at 1 day, 2 days, 3 days and 7 days timepoints respectively. Scalebar = 100μm.