

Non-Viral Xylosyltransferase-1 siRNA Delivery as an Effective Alternative to Chondroitinase in an *In Vitro* Model of Reactive Astrocytes

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Abstract

Reactive astrocytosis and the subsequent glial scar is ubiquitous to injuries of the central nervous system, especially spinal cord injury (SCI) and primarily serves to protect against further damage, but is also a prominent inhibitor of regeneration. Manipulating the glial scar by targeting chondroitin sulphate proteoglycans (CSPGs) has been the focus of much study as a means to improve axon regeneration and subsequently functional recovery. In this study we investigate the ability of small interfering RNA (siRNA) delivered by a non-viral polymer vector to silence the rate-limiting enzyme involved in CSPG synthesis. Gene expression of this enzyme, xylosyltransferase-1, was silenced by 65% in Neu7 astrocytes which conferred a reduced expression

of CSPGs. Furthermore, conditioned medium taken from treated Neu7s, or co-culture experiments with dorsal root ganglia (DRG) showed that siRNA treatment resulted in a more permissive environment for DRG neurite outgrowth than treatment with chondroitinase alone. These results indicate that there is a role for targeted siRNA therapy using polymeric vectors to facilitate regeneration of injured axons following CNS injury.

Keywords: polymer, transfection agent, dorsal root ganglia (DRG), co-culture, neurite outgrowth

Introduction

Injury to the central nervous system (CNS) results in an environment that is largely intractable to remodeling and repair, leading to permanent disruption of neuronal tracts and ensuing functional disability. Earlier work has shown that the CNS microenvironment is largely responsible for the restricted regenerative capacity following injury to the CNS [1-4]. The hallmark of this inhibition lies in the astrocytic glial scar, which has been studied since the early 1900s, as is illustrated in the work of Ramon y Cajal [5]. Since then, extensive work has been devoted to the study of the so-called reactive astrocytes (reviewed elsewhere[6]). In summary, after CNS injury, resident astrocytes become activated and proliferate, with a notable rearrangement in their structure, including increases in glial fibrillary acidic protein (GFAP) expression. Glial scarring ultimately is beneficial for survival of the injured CNS microenvironment, through (1) restricting leukocyte invasion, and (2) maintaining homeostatic balance [7,8]. Proliferation and hypertrophy of astrocytes, however, is also accompanied by production and deposition of an extracellular matrix, rich in proteoglycans, particularly chondroitin sulfate proteoglycans (CSPGs) [9-11]. The role that CSPGs play in confining axon growth has been well established since the early 1990s [2,12-14], and has thus become an appealing target for CNS repair. CSPGs represent a structurally diverse family of proteins with varied inhibitory properties on neurite growth. This diversity is imparted by the nature and number of the chondroitin sulphate glycosaminoglycan (CS-GAG) chains that attach covalently, via the linkage of xylose, to serine residues of the core proteins. This rate-limiting step

is catalyzed by the enzyme xylosyltransferase (XT), of which there are two known isoforms, XT-1 and XT-2 [15].

Multiple therapies have been proposed to target CSPGs post SCI, and hence limit their inhibitory effect. These therapies have generally employed either one of three different approaches. The first and most commonly used is to break down or degrade the inhibitory CS-GAGs using the bacterial enzyme chondroitinase ABC (ChABC) [4,16-18]. Despite being effective both *in vitro* and *in vivo*, however, there are certain limitations to the use of ChABC: (1) there is evidence that the remaining core proteins are still potentially functionally inhibitory to neurite outgrowth [19]; (2) ChABC is thermally unstable at 37°C and its activity is lost within 1-3 days, though this can be extended to weeks with stabilizing agents [20,21]; (3) it is likely that continuous infusion or repeated intrathecal administration would be required to have functional efficacy for larger injuries that are typically sustained in human SCI [22,23], ultimately carrying an increased risk of infection; and (4) ChABC may induce an immune response due to its bacterial origin. The second approach targets the biosynthetic pathway of CSPGs, such as the rate-limiting step catalyzed by the enzyme XT-1. In one study, the authors used DNA enzymes against XT-1, which despite showing good results *in vitro* and *in vivo*, has reportedly resulted in incomplete reduction of proteoglycan upregulation in the lesion core [24,25]. Using a slightly different concept, GAG chain polymerization was targeted using a plasmid-based short interfering RNA (siRNA) to decrease the expression of chondroitin polymerizing factor (ChPF) [26]. In a series of *in vitro* studies involving the Neu7 cell line, the authors were able to show that ChPF siRNA-treated Neu7 cells produced less CS-GAG chains, effectively making the Neu7 condition medium a less inhibitory one for axon growth, as evidenced by increased axon crossing in a boundary assay and increased neurite outgrowth in a neurite length assay. The third approach targeted the recently discovered CSPG receptor complexes (PTP σ and LAR). Receptor blockade has been achieved using inhibitory peptides, peptide mimetics, as well as transgenic deletion, and have led to various degrees of axonal sprouting, as well as locomotor and bladder functional improvement [27-29].

This study is aimed at investigating an alternative, non-enzymatic route to modulation of the glial scar that could infer greater efficacy than ChABC alone, and provide a more sustained knockdown than DNA enzymes, all the while targeting the biosynthetic pathway of CSPG at the rate-limiting step. As mentioned earlier, the rate-limiting enzyme in the synthesis of CSPGs is xylosyltransferase (XT), of which two isoforms exist. The XT-1 isoform is inducible by TGF- β 1 and is upregulated after injury [30]. It is hypothesized that targeting XT-1 using a siRNA coupled to a novel delivery vector could achieve greater knockdown efficiency of the enzyme, and a more sustained response, while minimizing the inflammatory response. This should translate into decreased production of CS-GAGs, and hence a more favorable environment for axonal regeneration.

Nucleic acids are susceptible to rapid degradation, siRNA even more so than plasmid DNA, potentially necessitating the use of a transfection vector [31]. A wide range of transfection vectors have been used to deliver nucleic acids to the CNS [32], so one objective of this study was to analyze the efficiency of XT-1 knockdown in Neu7 astrocytes by XT-1 siRNA delivered either without a vector (naked), with a cationic branched polymer poly(ethyleneimine) (PEI), or with a recently developed, cyclized knot transfection vector [33]. The second objective was to analyze whether this gene knockdown resulted in a more permissive environment for neurite outgrowth from adult dorsal root ganglion (DRG) cells, in (1) Neu7 condition medium, and (2) direct co-culture experiments of the two cell lines.

Materials and Methods

Materials

Neu7 cells were generously provided from James Fawcett [34]. Poly-l-lysine (PLL), Hank's balanced salt solution (HBSS), low glucose Dubecco's modified eagles medium (DMEM), fetal bovine serum (FBS) and the antibiotic mix of penicillin and streptomycin (P/S) were purchased from Sigma. Water used for all experiments was distilled and sterile-filtered before use (dH₂O).

Cell Culture

Neu7 cells were grown in low glucose (1000mg/L) DMEM supplemented with 10% FBS and 1% P/S using standard sterile techniques and incubated in 5% CO₂ at 37°C in a humidified atmosphere [35]. All experiments were performed with cells under passage number 6. 24 hours prior to the indicated study, cells were washed with HBSS and trypsinized, washed, and seeded in the appropriate wells for the specific study (indicated below). For experiments involving conditioned media, untreated or polyplex treated Neu7 cells were allowed to grow for 24 hours before their growth medium was collected. DRG neurons were obtained from adult rats. Cultures were prepared as previously described [36]. Briefly, thoracic or lumbar whole DRGs were de-sheathed, mechanically digested by passing through flame-narrowed Pasteur pipettes, and subsequently digested with 0.20% collagenase type I, followed by 0.25% trypsin, both in HBSS, for 15-20 minutes each, at 37°C. The cells were then preplated to allow non-neuronal cells to attach. DRGs with ~80% purity were then collected, washed, and seeded at a density of 1000 cells/cm² in Neurobasal (NB) medium supplemented with B27, 2mM L-Glut, and 1% P-S (Invitrogen).

XT-1 Knockdown Transfection Studies

24 hours prior to transfection, Neu7 cells were seeded in 12 well plates at a density of 10,000 cells/cm². Polyplexes (labelled as PD-E XT-1) were made up fresh prior to use as outlined previously and were added to the wells in serum-free media along with negative controls of the vector media, i.e. dH₂O (labelled as cells alone), and naked XT-1 siRNA (labelled as Naked XT-1) and a comparison vector PEI (labelled as PEI XT-1). Conditioned media was collected as described above.

Polyplex Characterisation

The cyclised knot polymer used to condense and deliver the siRNA was synthesised as previously reported [37], by deactivation enhanced atom transfer radical polymerisation developed to synthesize homopolymers [38,39] and copolymers [40,41] in a simple one-pot reaction. Briefly, this

polymer termed PD-E is comprised of a cationic moiety, 2-Dimethylaminoethyl methacrylate, to condense the negatively charged siRNA, and a branching unit, ethylene glycol dimethacrylate, to increase the efficiency of the vector [37] by forming the knotted structure. The polymer also contains a polyethylene glycol moiety intrinsically built into the structure during the reaction to reduce the cytotoxic effect of the cationic charge [42]. PD-E was dissolved in dH₂O at a concentration of 2 mg/mL and added to stock siRNA solution of 100 μM to form a final concentration of 50 nM per experiment and thus form condensed nanoparticles termed polyplexes, at a polymer to siRNA ratio of 6:1 (w/w). A 5 μL drop of this solution was placed onto a copper grid and allowed to dry for transmission electron microscopy analysis of the dehydrated size. To analyse the hydrated polyplex size, 100 μL of polyplexes were added to a cuvette for size determination in a zetasizer (Malvern Instruments), where this solution was diluted with a further 700 μL of water for surface charge measurements. A comparison vector, polyethyleneimine (PEI) was also used as received (25 kDa branched, Sigma) and dissolved in dH₂O at a concentration of 2 mg/mL and added to siRNA, to form polyplexes at a polymer to siRNA ratio of 6:1 (w/w).

Construction of the XT-1 siRNA

XT-1 siRNAs were obtained from Eurofins MWG operon (Ebersberg, Germany). Five candidates were initially tested for validation on Neu7 cells using RT-PCR, and the following sequence was chosen: Sense 5'-[GAUAACAACCUGCGCAUCACCAAUUGG], Antisense 5'-[CCAAUUGGUGAUGCGCAGGUUGUUAUC]. siGLO Cyclophilin B siRNA was used to assess cell uptake and ON-TARGETplus siRNA as a non-targeting (NT) control in all other experiments (both Dharmacon, USA).

Axon Growth in Conditioned Media

DRG were extracted as detailed above. DRG cells were seeded in PLL-coated 8-well glass chamber slides (Nunc Lab-Tek II), at a density of 1,000 cells/well and left for 24 hours to attach, prior to experimentation. The media was then removed, cells washed with HBSS, and the conditioned

media from the XT-1 knockdown experiment was added to the chamber wells at a 1:1 ratio with the DRG culture medium. The cells were then left in the conditioned media for a further 48 hours. Prior to fixing, the cells were washed three times with HBSS and fixed at room temperature with 4% formaldehyde solution in phosphate buffered saline. A monoclonal β III tubulin antibody (Tuj1; Abcam) was used to stain neurons as described previously [43]. Finally, the chamber walls were removed and the slides were mounted using VECTASHEILD[®] (Vector Labs, UK) mounting medium containing the nuclear counter-stain DAPI. A fluorescent microscope (Olympus IX81, Mason Technologies, Dublin, Ireland) was used to obtain images (10x) from a minimum of 30 random fields of view by a person blinded to the experimental groups. The β III tubulin stain was measured at an emission wavelength of 488 nm and the DAPI filter for the nuclear stain at 455 nm. The longest neurite from each cell body was identified and measured using the ImageJ plug-in NeuronJ by a person also blinded to the experimental groups {Meijering:2004ip}. These groups were repeated in triplicate and an average of these longest neurites (minimum of 30) was calculated for each group.

XT-1 Knockdown Analysis

Total RNA was extracted using the RNeasy Mini Kit (Qiagen) according to the manufacturer's protocol. Total RNA quantity and purity were determined using an ultraviolet spectrometer (NanoDrop ND-1000 Spectrophotometer). Reverse transcription (RT) was performed using the TaqMan RT system according to the manufacturer's protocol (Applied Biosystems, Switzerland). Gene transcription was examined using quantitative real-time polymerase chain reaction (qPCR). Reactions were performed and monitored using an ABITM 7500 sequence detection system (Applied Biosystems) using the TaqMan[®] gene expression Master Mix (Applied Biosystems). mRNA levels were normalized to GAPDH. The primer sequences were as follows: XT-1 forward 5'-[GTCTGTCCACCTCTACTTCC], reverse 5'-[TCATCCATGTCTCCAGTGTC].

XT-1 and CSPG Expression in Neu7 Cells

Neu7 cells were cultured as outlined above and seeded in PLL-coated 8-well chamber slides at 10,000 cells/cm². 24 hours post attachment, cells were treated with either Naked DNA, PD-E XT-1 siRNA, or ChABC enzyme. The latter was used as a control, as it has been shown to reduce expression of CSPG in Neu7 cells. Two different ChABC treatment conditions were used, in order to differentiate between early and late effects of ChABC on CSPG expression; the first termed ChABC 'early', involved treating Neu7 cells with the enzyme at the 24-hour mark, at the time the polyplexes are added. In the second condition, termed ChABC 'late', previously untreated cells were exposed to the enzyme 12 hours later, i.e. 36 hours post seeding. All groups were incubated for a further 18 hours whereafter they were washed and fixed. After fixation with 4% paraformaldehyde, chamber wells were washed with phosphate buffered saline (PBS) and then incubated for 30 minutes in blocking buffer (5% normal goat serum and 0.1% bovine serum albumin in PBS). Goat anti XT-1 (Santa Cruz) and mouse anti CS-56 (Sigma) primary antibodies were diluted 1:200 in blocking buffer for overnight incubation at 4°C. Secondary antibodies containing an Alexa Fluor anti-goat 594 (XT-1) or Alexa Fluor anti-mouse 488 (CS,56) were used. Slides were mounted using VECTASHEILD[®] mounting medium containing DAPI counterstain. Images were obtained at 40x magnification on an Olympus IX81 fluorescent microscope (Mason Technologies, Dublin, Ireland).

Cell Viability Analysis

Neu7 cells were incubated with polyplexes, vehicle or naked siRNA as described above. The cells were immediately washed 3 times with HBSS and incubated in a 10% solution of alamarBlue[®] in HBSS for 3 hours. Blank analysis was performed by incubating both HBSS and alamarBlue[®] solution in empty wells for the same period. The blue colour of the alamarBlue[®], when metabolised, turns into a pink colour, which is used to analyse the metabolic activity of the cells. 100 µL of the cell supernatant was transferred to clear, flat-bottomed 96 well plates for photometric analysis in a Varioskan multiwell plate reader (Thermo Scientific, Ireland) and analyzed as per the manufacturer's protocol. All values were normalized to untreated cells, depicted as 100% viable. Experiments were performed in quadruplicate with average values plotted.

Axon Growth in Co-culture Assays

Neu7 cells were treated with either Naked siRNA, PD-E XT-1 siRNA, or ChABC 'early'. 12 hours post treatment, the media was changed and after a further 18 hours in culture, freshly extracted DRG cells were added to the Neu7 cells at a density of 1,000 cells/cm². For the ChABC 'late' group, the enzyme was added at the same as the extracted DRGs. After a further 30 hours, the cells were washed, fixed, and stained with β III tubulin as described above. 10x fluorescent images were obtained of at least 30 random points by a person blinded to the study groups (performed in triplicate). NeuronJ was used to measure neurite length.

Statistical Analysis

Statistical analysis was performed using GraphPad Prism® Software. For all studies a One Way ANOVA was performed using a Tukey's post-hoc analysis and $P < 0.05$ was considered a statistically significant difference from the cells alone control group.

Results

The PD-E knot polymer, used as a non-viral transfection agent, was able to condense the XT-1 siRNA into nanoparticles termed polyplexes (Figure 1). Polyplexes formed at a 6:1 polymer to siRNA ratio were then characterised by dynamic light scattering using a zetasizer. The hydrated diameter of these polyplexes was 301nm (+/- 83nm) and the surface charge was slightly cationic at only 5mV (+/- 0.7mV) (see supplementary information). However, when this polyplex dispersion was placed onto a copper TEM grid to dry, the dehydrated diameter was much lower, with typical values being between 50-90nm (Figure 1b). This is in agreement with the concept that the knot polymer forms loose complexes with nucleic acids [33].

Figure 2a shows that both polymeric vectors (PEI and PD-E) were able to mediate lower XT-1 messenger RNA levels as quantified by PCR, than that of cells alone or using the siRNA without a vector (Naked XT-1). The knot polymer showed high nucleic acid delivery efficiency exhibiting a

65% knockdown efficiency. The knockdown observed when using the knot polymer was significantly greater than the no treatment group (termed 'cells alone'), Naked XT-1, and the PEI vector group. When the cell viability was analyzed for the same treatment groups, naked XT-1 and PD-E XT-1 showed no adverse effect at the concentration and ratio used. However, PEI XT-1 showed a reduction in viability to 87.5%.

To see if XT-1 knockdown could produce a functional effect, DRG neurite outgrowth was assessed using Neu7-conditioned media. Neu7 cells are inherently inhibitory to neurite outgrowth from DRGs. This inhibition is mediated largely by the increased production of CSPGs, a large portion of which is extruded into the media. This inhibitory effect was alleviated, at least partially, when Neu7 cells were pre-treated with ChABC. When DRGs were seeded and incubated in conditioned media from the different treatment groups, a difference in neurite length was observed. Only the PD-E XT-1 group showed a statistically significant improvement in the neurite length, when compared to cells alone, naked XT-1, and PEI XT-1 (Fig 2c).

To determine whether this functional effect was indeed due to the knockdown of XT-1, fluorescent immunocytochemistry was conducted using antibodies against CS56 and XT-1. Chondroitinase digests the glycosaminoglycan chains of CSPGs that are prohibitory to neurite growth and thereby encourages plasticity. A decrease in CS-56 levels was observed as an acute effect of ChABC 12 hours post treatment. However, no reduction was observed 48 hours after ChABC was withdrawn, whereas PD-E XT-1 treated cells at this timepoint showed a significant reduction in CS compared to control groups (Figure 3b - representative images d and f). In accordance with this, levels of XT-1 were decreased in PD-E XT-1 treated wells compared to the ChABC treatment group.

To test if this reduction in CSPG could result in a more permissive environment for DRG neurite growth, DRGs were co-cultured on top of treated Neu7 cells. A co-culture system as such has the added benefit of testing both matrix-bound and extruded forms of CSPG. ChABC treatment at the time of adding polyplexes had no effect on neurite outgrowth thus indicating that repeat treatments

would be necessary. Although naked XT-1 siRNA had no effect compared to cells alone, polyplex delivery of XT-1 siRNA in the PD-E XT-1 treatment group allowed significantly longer neurite outgrowth, thus creating a more permissive environment for growing axons.

Discussion

Some recent clinical trials have involved the use of siRNA either with, or without the use of a vector (reviewed in [44]). For respiratory based applications, where repeat aerosol administrations are possible, siRNA has been delivered without an accompanying vector [45,46]. However, for systemic siRNA administration a delivery vector has been used [47]. This vector was composed of three parts: 1) a cationic unit to bind the siRNA, 2) a targeting ligand to enhance delivery, and 3) a hydrophilic PEG unit to improve stability [47]. Similarly, the polymer vector used in this study was composed of three parts but all were combined in a simple one-pot reaction. 2-dimethylaminoethyl methacrylate (82%) served as the cationic moiety for complexing the siRNA, ethylene glycol dimethacrylate (10%) facilitated the knot structure and PEG (8%) was introduced to reduce the vector's toxicity. This polymer vector has previously been shown to deliver DNA effectively to neuronal cells [33], and in this study greatest XT-1 knockdown was achieved using this vector without inducing toxicity. This knockdown could potentially be further enhanced by functionalising the free vinyl groups on the polymer structure with specific neuronal cell targeting ligands such as TET1 [48] or rabies virus glycoprotein RVG-29 [49].

Following CNS injury, glial cells migrate to the injury site where they become hypertrophic and proliferate in a that is loosely termed as reactive. Their reactivity is a reflection of their ensuing function in the vicinity of the injury site which includes the excessive production of ECM components and chondroitin sulfate proteoglycans. Whereas some CSPG production in the normal CNS is believed to originate in the neuronal cell bodies, it is the reactive astrocytes that lead the production of CSPG following injury to the CNS [2,50]. There have been many proposed mechanisms for GAG-induced inhibition of axon growth. Possibly the earliest and most basic of

mechanisms is steric hindrance of growth promoting adhesion molecules, such as laminin and integrins. CSPGs can also attenuate integrin signaling, through their highly charged GAG moieties [51,52]. Additional inhibition of axon growth occurs via the interaction of CSPGs with chemotropic guidance cues, such as Sema5A and Sema3A, which in turn potentiates their inhibitory action on axon guidance and migration [53,54]. Recently, two studies have investigated two potential transmembrane proteins, namely PTP σ and leukocyte common antigen related (LAR) phosphatase, which can bind with high affinity to CSPGs and subsequently mediate their inhibitory effect [27,55,56].

In this work, we targeted the CSPG synthesis step where glycosaminoglycan (GAG) chains are attached on the serine residues of the core protein via a tetrasaccharide sequence of xylose-galactose-galactose-glucuronic acid (Xyl-Gal-Gal-GlcA). This reaction begins in the endoplasmic reticulum (ER) by the addition of the tetrasaccharide to serine residues via two enzymes: xylosyltransferase 1 and xylosyltransferase 2 (XT-1 and XT-2). We hypothesized that effective silencing of this linking step could reduce the inhibitory nature of the reactive Neu7 astrocytes and allow a more permissive environment for neurite outgrowth. The large discrepancy observed between the ChABC early and late treatment groups highlighted the short-acting nature of this enzyme which is thermally unstable. Considering the chronic nature of the glial scar it indicates that repeat administration may be necessary and the complications that entails. The PD-E XT-1 treatment group showed sustained suppression of CS-GAGs and increased neurite growth compared to ChABC. However, as siRNA mediated silencing is transient in nature; an alternative delivery strategy should be sought before undertaking *in vivo* analysis of PD-E XT-1. Repeat administration in the CNS may prove difficult, invasive or impossible, so a method of prolonged release of siRNA could be envisaged through the use of siRNA functionalized biomaterial scaffolds [57]. In this way, extracellular matrix scaffolds designed for physically directing neuron growth [43] could be further functionalized with siRNA or better still siRNA-polyplexes.

Conclusions

This study aimed to investigate the use of siRNA to silence xylosyltransferase-1, one of the rate-determining enzymes in the synthesis of CSPGs. Since CSPGs are neuroinhibitory in nature, it was hypothesized that knockdown of this enzyme would result in a more permissive environment for neurite outgrowth. A newly developed and highly effective cyclized knot transfection vector was employed to deliver the siRNA to Neu7 astrocytes. This resulted in a 65% knockdown of XT-1 expression as determined by PCR without affecting Neu7 viability. This knockdown resulted in a more permissive environment for DRG neurite outgrowth as determined by conditioned media and co-culture experiments. This siRNA-based technology may complement or provide an attractive alternative to current developments in chondroitinase ABC-based modulation of the glial scar that forms post-injury to the central nervous system.

Acknowledgements

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Figures

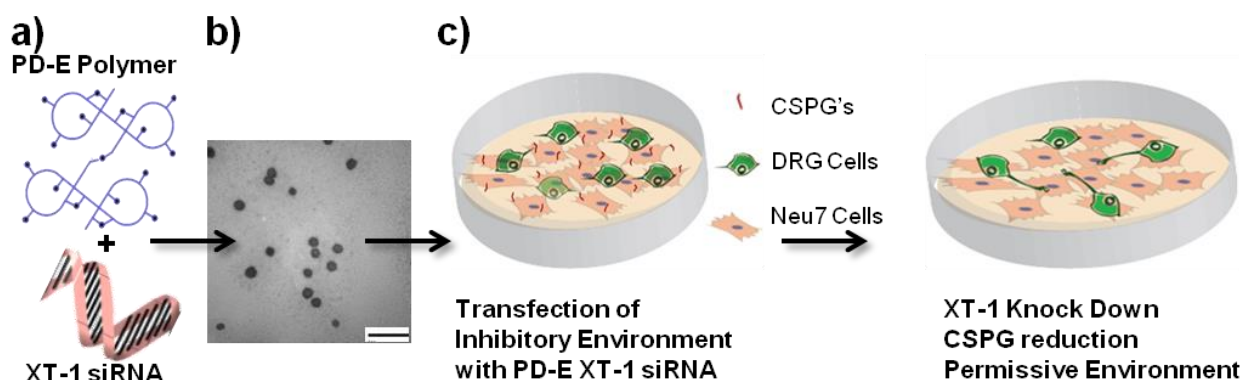


Figure 1

Fig 1 Schematic representation of the cyclized knot structured PD-E Polymer and XT-1 siRNA (a) which when combined form nanoparticle polyplexes as shown by transmission electron microscopy (scale bar = 500nm)(b). When added to a bed of Neu7 astrocytes, these polyplexes mediate XT-1

knockdown and suppress CSPG production, allowing a more permissive environment for dorsal root ganglia neurite outgrowth

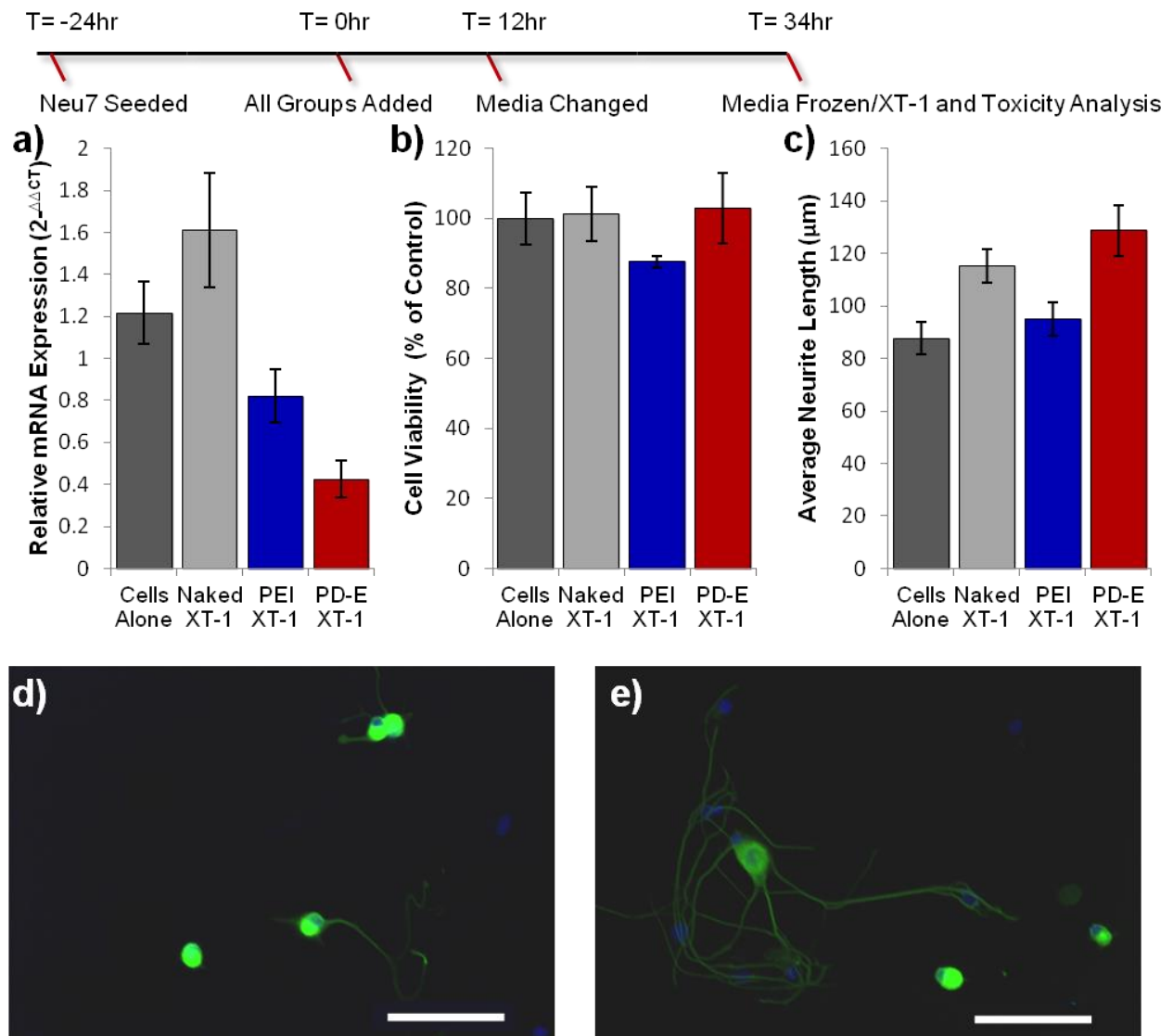


Figure 2

Fig 2 Assessment of XT-1 knockdown (a), Neu7 viability (b) and effect of conditioned Neu7 media on DRG neurite outgrowth (c) via XT-1 siRNA without a vector (Naked), with a commercially available vector (PEI) and with the cyclized knot vector (PD-E). Despite large variability within groups, PD-E XT-1 mediates significant XT-1 knockdown, and longer DRG average neurite length than the cells alone control, without inducing toxicity. Representative images of β III tubulin stained DRG cells cultured in the conditioned media of cells alone group (d) and PD-E XT-1 group (e).

Scale bars represent 100 μm , $n=4$, * indicates statistically significant difference from cells alone group, where $P<0.05$ (one way ANOVA).

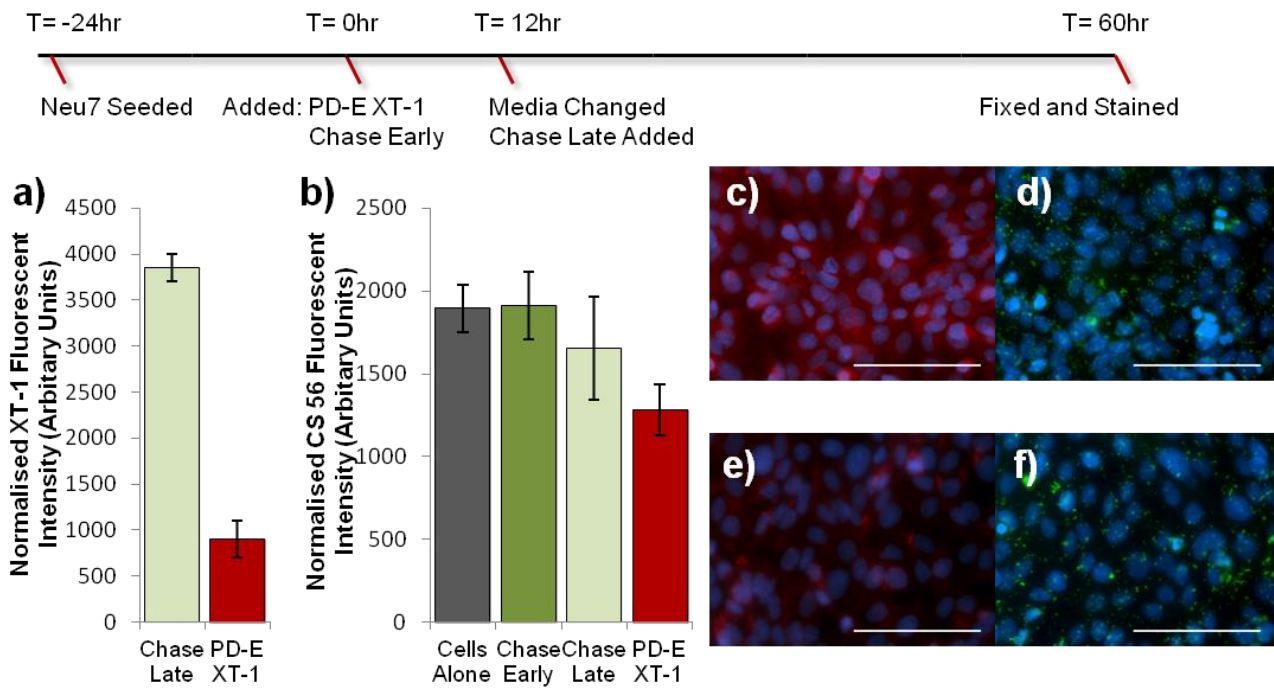


Figure 3

Fig 3 Treatment of Neu7 astrocytes with XT-1 siRNA delivered via PD-E results in a lower fluorescent intensity of XT-1 staining (a) and CS,56 staining (b) compared to ChABC controls with representative images of ChABC Late group (c-d) and PD-E XT-1 group (e-f) showing little difference in CS,56 staining (d and f) but a more marked difference in XT-1 staining (c and e).

Scale bars represent 100 μm , $n=4$, * denotes statistical significant difference from control group (ChABC Late (a) and (cells alone (b)), where $P<0.05$ (one way ANOVA).

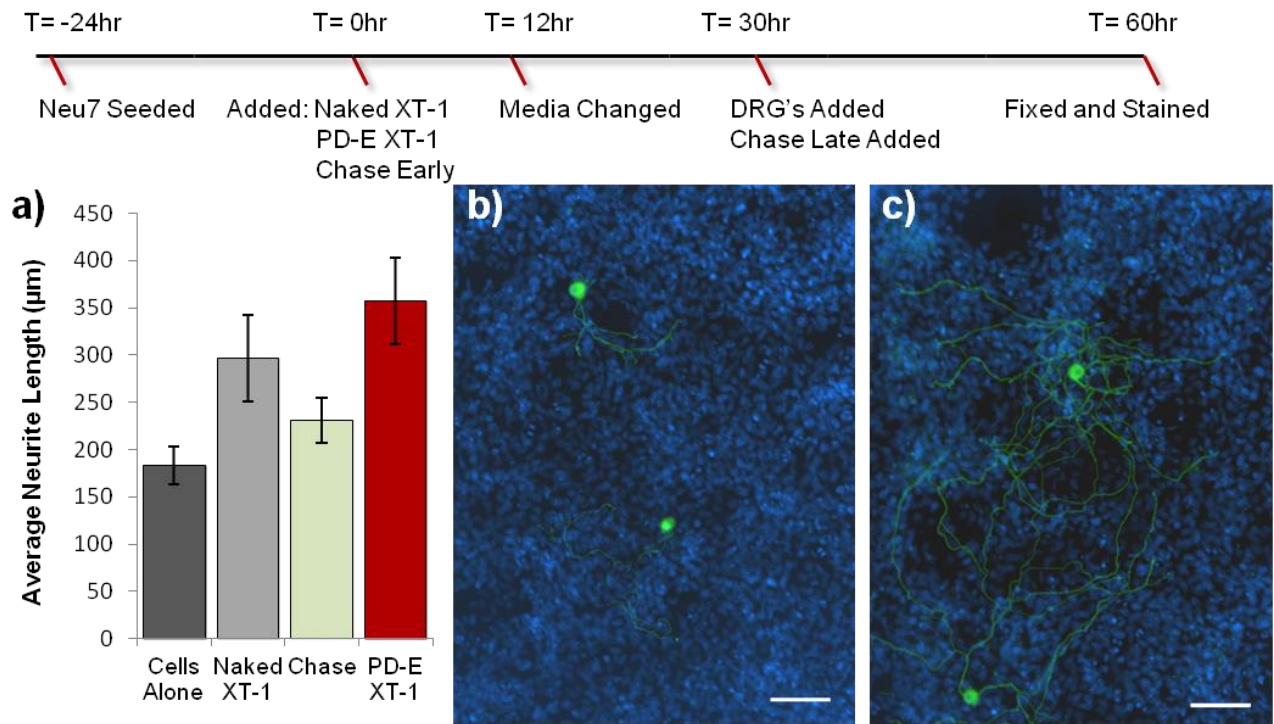


Figure 4

Fig 4 Effect of Neu7 astrocytes on the neurite outgrowth of co cultured DRGs as measured by the average length of the longest neurite (a) showing the beneficial effects of PD-E XT-1 treatment. Representative images of DAPI counterstained Neu7 cells and β III tubulin stained DRGs for Cells Alone group (b) and PD-E XT-1 group showing long extending neurites (c). Scale bars represent 100 μm , n=4, * denotes statistical significant difference from cells alone group, where $P < 0.05$ (one way ANOVA).

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