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European Journal of Pharmaceutics and Biopharmaceutics 61 (2005) 126–133
European Journal of Pharmaceutics and Biopharmaceutics
www.elsevier.com/locate/ejpb

Research paper

Delivery of a lentiviral vector in a Pluronic F127 gel to cells of the central nervous system

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Received 6 August 2004; accepted in revised form 20 June 2005
Available online 8 September 2005

Abstract

Lentiviral vectors have been demonstrated as efficient tools for gene delivery to the CNS. We describe a novel approach for vector delivery using the thermoresponsive Gel, Pluronic F127 as a carrier. A HIV-1 lentiviral vector expressing GFP was contained in various concentrations of gel (15, 30 and 40%) and applied to cultures of 293T cells. FACS analysis of cells transduced with 8 ng of lentiviral vector revealed a similar transduction efficiency for each Gel concentration compared to vector added to cells without PF127. Primary Rat CNS mixed glial cultures were also transduced with lentiviral vector in 15% Pluronic F127 and results demonstrated a similar transduction efficiency of astrocytes compared to virus without gel and no evidence of cell toxicity or death. Stereotaxic delivery of viral vector in 15% PF127 to the rat brain resulted in transduction of cells, predominantly astrocytes close to the injection site. Pluronic F127 gel delivery of viral vectors to the CNS may provide a platform for localised release particularly in areas of brain or spinal cord injury.

Keywords: Lentiviral vector; Pluronic F127; Viral vector delivery; Central nervous system

1. Introduction

Pluronics, also known as poloxamers are a series of commercially available block co-polymers with pharmaceutical applications. Pluronic F127 is an ABA-type triblock copolymer consisting of polyoxyethylene (A) and polyoxypropylene units (B). PF 127 in aqueous solution at concentrations of 15–20% and higher exhibits the unique property of reversible thermal gelation [1]. When the temperature of an aqueous solution of F127 is increased, the co-polymer molecules aggregate into spherical micelles, which contain a dehydrated polyoxypropylene (PPO) core and an outer shell of hydrated polyoxyethylene (PEO) chains [2]. This unique property of being liquid at 4 °C and in a semi solid gel at room or body temperature provides an attractive platform for slow release of drugs. The gelation mechanism of pluronics is thought to occur via micelle formation at a critical temperature resulting in dehydration of the PPO block [25] with possible packing of micelles at increased temperature [26]. The micellar nature in an aqueous environment has been shown to be suitable for the incorporation of both hydrophilic and hydrophobic drugs [3] and to prolong drug release [27,28]. Delivery of proteins such as interleukin-2 and urease [4] has also been described and recently plasmid DNA in a photo-cross-linked pluronic hydrogel [29]. Poloxamer gels display low toxicity and low clinically useful doses do not increase serum triglycerides and cholesterol in animal models [5]. Medical uses of pluronic F127 have included the controlled delivery of drugs to the eye [6], nasal passage as well as parenteral and subcutaneous administration [7].

Viral vectors have proved to be an efficient means for gene delivery both in vitro and in vivo. Several studies have described the co-delivery of adenoviral vectors with a poloxamer 407 gel to vascular cells in vitro [8] and to

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doi:10.1016/j.ejpb.2005.06.006

the carotid artery of the rabbit [9]. Poloxamer 407 has been reported to increase the transfection efficiency and reduce the incubation time for adenoviral mediated gene transfer. Lentiviral vectors based on Human Immunodeficiency Virus Type 1 (HIV-1) have demonstrated effective gene delivery to non-dividing cells particularly cells of the central nervous system (CNS). Several characteristics of lentiviral vectors make them attractive for gene delivery, such as an ability to integrate into the host chromosome and provide long-term stable gene expression, a large cloning capacity of up to 8 kb and a lack of a host immune response due to the non-expression of viral genes in transduced cells [10]. Lentiviral vectors based on human immunodeficiency virus type 1 (HIV-1) pseudotyped with the vesicular stomatitis virus (VSV-G) envelope have been shown to transduce neurons in brains of rodents [11] nonhuman primates [12] and in oligodendrocyte lineage cells in the rat spinal cord [13]. Combining a lentiviral vector and a pluronic-based gel has not been previously reported and may have applications in gene delivery to the CNS particularly in areas of traumatic injury to the brain or spinal cord. Indeed, the application of hydrogels to axon regeneration has been demonstrated with a biocompatible porous hydrogel of poly [*N*-(2-hydroxypropyl)methacrylamide] (PHPMA) in a model of spinal cord injury to the cat [17].

In this study we assessed the ability of pluronic F127 gel to encapsulate an HIV-1 based lentiviral vector expressing GFP and transduce 293T cells and primary mixed glial cultures from rat. We also report the stereotaxic injection of pluronic F127 gel containing lentiviral vector expressing GFP into the thalamic region of the rat brain and transduction of astrocytes.

2. Methods

2.1. Pluronic gel preparation

Pluronic F127 (Sigma) was dissolved in PBS overnight at 4 °C on a roller to achieve various concentrations of 15, 30 and 40% (W/V). Once dissolved the solutions were stored at 4 °C.

2.2. Lentiviral vector preparation

A HIV-1 lentiviral vector was produced by a four-plasmid calcium chloride co-transfection of 293T cells. Seven micrograms of a gene transfer vector expressing GFP and containing the HIV-1 central DNA flap region cloned into a unique Hpa site upstream of the CMV promoter (HIV-cPPT-GFP), 7 μg of a gag-pol expression plasmid (delta 8.91, a kind gift of D Trono), 3 μg of a HIV-1 Rev expression plasmid and 3 μg of the vesicular stomatitis virus-G envelope were added to a mixture of BBS and Calcium chloride. After 30 min incubation the solution was added dropwise to a 10 cm dish of 293T cells at 70–80% confluency grown in DMEM and 10% FCS. The transfected cells were incubated at 37 ° C and 3% CO_2 overnight. The media was then replaced and cells were incubated at 37 °C and 5% CO_2 for a further 48 h. The cell supernatant containing recombinant viral vector was ultracentrifuged for 2 h at 50,000 × *g*. The viral vector pellet was resuspended in 50 μl of PBS containing 1% BSA and stored at −70 °C.

2.3. Lentiviral vector Titration

The recombinant viral vector preparation was titred by measuring reverse transcriptase activity using a commercially available kit (Cavidi Tech) with HIV-1 reverse transcriptase (RT) standards. Lentiviral reverse transcriptase levels were expressed as ng/μl.

2.4. FACs analysis of lentiviral vector transduced cells

293T cells were transduced with known amounts of lentiviral vector as calculated by the RT assay. Viral vector diluted in DMEM media containing 6 μg/ml of polybrene (Sigma) was applied to cell monolyers overnight with media being replaced the following day. GFP expression was generally observed by microscopy 48 h post transduction. Transduced cells were prepared for flow cytometry by trypinsinisation, centrifuged for 5 min for 2000 × *g* and treatment with 3% paraformaldehyde for 10 min. The cells were centrifuged again at 2000 × *g* for 5 min and resuspended in 1 ml of PBS for FACs analysis using a FACS calibur instrument (Beckton Dickinson).

2.5. Glial cell culture preparation

All cell culture reagents were purchased from Invitrogen (Paisley, UK), unless otherwise stated. 293T cells were cultured in DMEM containing 10% FCS. Primary glial cultures were prepared from the brains of newborn rats >3 days old, as described previously [18].

Mixed glial cultures were derived from these cells, once they were confluent, by first washing them with EDTA/PBS and then adding trypsin for 2–5 min to remove the cells from the surface. DMEM containing 10% FCS and 1% PSF was then added to neutralise the trypsin and the whole suspension spun at 1000 rpm for 5 min. The supernatant was removed and the cells resuspended in 10% FCS/ DMEM and plated onto Poly-D-Lysine (PDL) coated coverslips in 4- or 24-well plates. This media was changed after 4 h and then every 2 days post plating for the duration of the experiments.

The media was changed 24 h after transduction and the cells left for a further 48 h (72 h in total) before they were fixed. Four percent paraformaldehyde was added for 15 min and then the cells were washed and stored in PBS until they were ready to be stained.

2.6. Surgical procedures

All surgical procedures were done in accordance with Home Office (UK) regulations on controlled procedures. To compare delivery of HIV-1 vector expressing GFP in 15% PF 127 and in PBS +0.1% BSA, injections were performed into the thalamic region of the brain of adult (~220 g Sprague–Dawley) rats. Fifteen percent PF 127 or PBS +0.1%BSA was used as a control. Injections were performed using a Hamilton Syringe with a pulled glass micropipette attached to the end, which was siliconised to aid passage through the brain and decrease damage. The rats were anaesthetised using Avertin (5 g tribromoethanol with 20 ml of ethanol and 3 ml amyl alcohol, 2-methylbutan-2-ol in 250 ml 0.9% saline solution) at 1 ml/100 g bodyweight i.p. They were placed into a stereotaxic frame and the skull was exposed and Bregma located. A 1 mm hole was drilled through the skull at −2.9 mm laterally and −2.8 mm anteriorly. Dura was located and the needle lowered −5.2 mm ventrally until in position. Injections to the thalamus area of the rat brain were performed over several minutes and the needle left in position for 5 min before being removed and the scalp was sutured. Four percent glucose—0.18% saline (Aquapharm, 5 ml/flank s.c.) was administered and they were then left to recover in a heated box. Paracetamol was provided in drinking water (1 mg/ml) for 3 days post-operation.

Rats were sacrificed 3 days post-injection using a lethal dose of sodium pentobarbitone (Euthatal) at 0.3 ml/100 g bodyweight i.p. and were then perfused through the heart with 300 ml cold PBS pre-wash followed by 300 ml cold 4% paraformaldehyde. The brains were removed and fixed in 4% paraformaldehyde for 4 h before being transferred to a 30% sucrose solution in PBS for cryoprotection. 30 μm coronal sections were then cut on a freezing sledge microtome and stored in PBS with 0.05% sodium azide.

2.7. Immunohistochemistry.

Lentiviral vector transduced mixed glial cultures on coverslips were blocked using 3% goat serum in TXTBS (0.2% Triton X-100, Sigma, in Tris buffered saline) for one hour. Monoclonal anti GFAP (Sigma, 1:500) and polyclonal goat anti rabbit GFP (Molecular Probes, 1:1000) were used diluted in TXTBS with 1% normal goat serum (NGS) for 2 h. Coverslips were then washed at least 3 times in TBS over a 30 min period before secondary antibodies were added with 1% NGS in TXTBS for 1.5 h. anti-mouse Alexa (Molecular Probes, 1:500) and biotinylated goat anti-rabbit (Amersham Biosciences, 1:500). Coverslips were washed again for half an hour in TBS and then Streptavidin-FITC was used to visualise the GFP staining (Serotec, 1:100) in TBS with 1% NGS and Bis-benzamide (Sigma, 1:5000) to visualise the cell nuclei. Fluorosave Reagent (Calbiochem) was used to mount the coverslips on to slides.

Double staining was also carried out on the brain sections. Again polyclonal anti-GFP (Molecular Probes, 1:1000) was used in conjunction with either monoclonal anti-GFAP (Sigma, 1:500), monoclonal antibody raised against CD11-b clone OX-42 (Serotec, 1:400) was used to identify reactive macrophages and microglia and a monoclonal anti-NG2 (Hybridoma conditioned media, 1:10) to identify oligodendrocyte precursor cells.

Primary antibodies were left on overnight at room temperature, after blocking in NGS at 3% in TXTBS (0.2% Triton-X-100 in tris-buffered saline). Brain sections were washed in tris-buffered saline (TBS) and the secondary antibodies applied in TBS with NGS for 4 h at room temperature. Anti-mouse Alexa (Molecular Probes, 1:500) and biotinylated goat anti-rabbit (Amersham Pharmacia Biotech, 1:500) secondary antibodies were used. The sections were washed again in TBS. Streptavidin -FITC (Serotec, 1:100) was applied for 1.5 h with the nuclear Hoechst's stain bis-benzamide (Sigma, Hoechst no. 33258, 1:5000). Sections were then washed in TBS followed by tris-buffered non-saline (TNS) and mounted using flurosave reagent (Calbiochem).

2.8. Cell counts and statistical analysis

In vitro cell counts were performed from one edge of the coverslip all the way across to the other, horizontally and vertically. A 0.5 mm^2 area was counted every 1.5 mm across the coverslip. In total 18 squares of 0.5 mm^2 area were counted for each coverslip. Each virus transduction was performed in duplicate, a total of 36 cell counts were averaged to calculate the mean and standard error of the mean for cell counts per 0.5 mm^2. Total cell count was achieved by counting cells stained with bis-benzamide, GFP +ve and GFAP positive cells were also counted following combined immunostaining. GFP and GFAP double-labelled cells were also counted and the percentage of GFP+ve and GFAP+ve cells of the total GFP+ve cells was calculated.

2.9. Cell transduction

Twenty to 2.5 ng of lentiviral vector in 20 μl of PBS was added to an equal volume of pluronic gel ranging in concentration from 30 to 80%, mixed and stored on ice, to achieve a final PF 127 concentration of 15–40%. The solution was then added directly to culture media overlying 293T or glial cells and incubated at 37 °C for 48–72 h. GFP expression in transduced 293T cells was analysed by FACS. FACS analysis was performed using BD Biosciences FACScalibur instrument on vector transduced cells which were first trypsinised with and fixed with 3% paraformaldehyde. Mixed glial cells were transduced with varying concentrations of lentiviral vector with or without 15% pluronic F127. Transduction efficiency of glial cells was assessed by counting GFP positive cells immunostained with a monoclonal antibody to GFP. Lack of GFP

expression at early time points after transduction confirms that GFP detection at 72 h was vector mediated and not passive protein transfer.

3. Results

3.1. Transduction of 293T cells and rat mixed glial cultures

FACS analysis of 293T cells transduced with lentivirus vector demonstrated that similar levels of vector transduction were achieved with and without.the pluronic gel (Fig. 1). Importantly no decrease in transduction efficiency was observed with pluronic and no toxic effects on the cells were observed. The number of 293T cells transduced by 8 ng of lentiviral vector was similar when increasing pluronic gel concentrations of 15, 30 and 40% were used compared to the same amount of lentivector without PF127. Transduction of mixed glial cultures with GFP expressing lentivirus vector resulted in strong expression 72 h post-transduction with vector diluted in either tissue culture medium or pluronic F 127 gel (Fig. 2A and B).

Rat mixed glial cultures were transduced with a range of lentivirus vector concentrations from 20 to 2.5 ng diluted in culture media (Fig. 3A) and in 15% pluronic F127 gel (Fig. 3B). At higher concentrations of input lentivirus vector (20 and 10 ng) 97 and 73% of cells were transduced without pluronic gel compared to 78 and 51% of cells transduced for the amounts of lentiviral vector in 15% PF 127. At 5 ng input of lentivirus vector, the transduction efficiency was relatively similar with 56% of cells transduced without PF127 and 60% with 15% PF 127. A slight increase in the number of cells transduced was seen with an input of 2.5 ng of lentivector in 15% PF127 (40%) compared to 32% for lentivirus vector added to glial cultures without PF127. The number of astrocytes specifically transduced by the lentivirus vector was assessed by co-immunostaining GFP

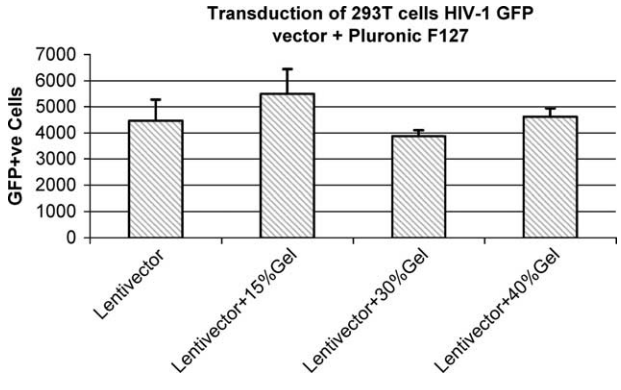

Fig. 1. FACS analysis of 293T cells transduced with a HIV-1 based lentivirus vector expressing GFP diluted in tissue culture media or increasing concentrations of pluronic F127 gel. Results expressed as number of GFP positive cells, for transductions performed in duplicate (lentivector in DMEM) and in triplicate (lentivirus vector in pluronic F127 gel).

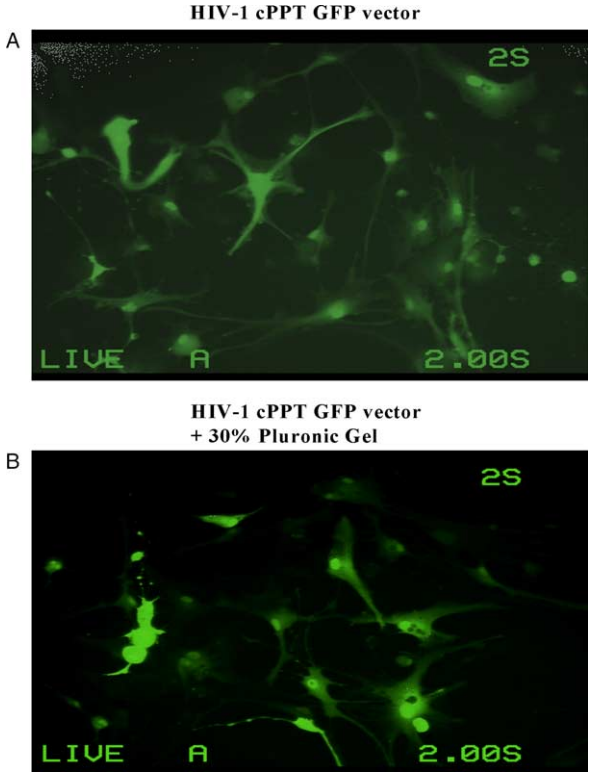

Fig. 2. GFP expression in rat primary mixed glial cultures following lentiviral vector transduction in cell culture media (A) or 15% pluronic F127 gel (B).

positive cells with an antibody to glial fibrillary acid protein (GFAP). With a lentivirus vector input of 20 ng in 15% PF127, 90% of the GFP positive cells were astrocytes compared to 76% without PF127. At the lower viral vector amounts, the number of astrocytes transduced was similar with or without PF127. The presence of pluronic gel had no toxic effect on the mixed glial cultures as demonstrated by a similar cell count for non transduced cells (control) and transduced cells.

3.2. Stereotaxtic delivery of lentiviral vector to the rat brain

Injection of a small volume (2 μl) of lentiviral vector (20 ng of Reverse transcriptase) into the thalamus area of the rat brain resulted in transduction of cells and expression of GFP, 3 days post injection. Confirmation of GFP expression by immunohistochemistry revealed transduction of astrocytes as the predominant cell type by cell morphology particularly at ×40 magnification (Fig. 4A and C).

Co-delivery of 2 μl (20 ng of HIV-1 RT) lentiviral vector plus 15% pluronic gel by stereotaxic injection into an area close to the sub-ventricular zone resulted in expression of GFP as detected by immunohistochemistry with DAB staining, 72 h post-transduction. GFP positive cells were detected some distance away from the injection site (Fig. 4B and D), however, damage to the brain parenchymal tissue at

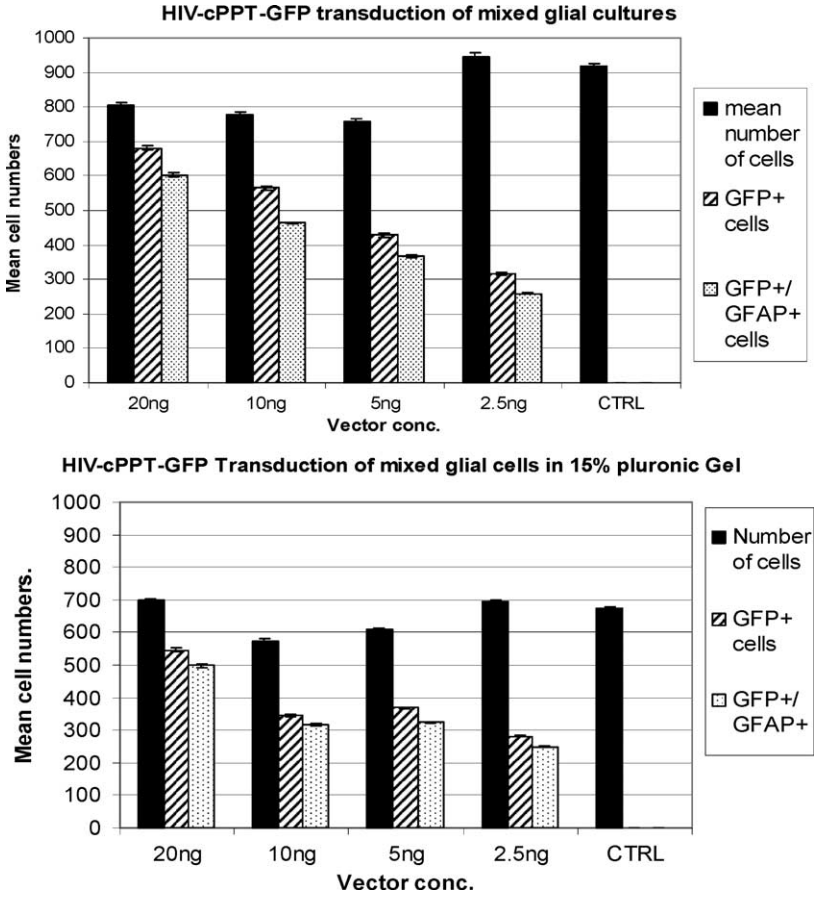

Fig. 3. (A) Quantitative assessment of lentivirus vector transduction efficiency of primary mixed glial cells with lentivector diluted in DMEM. (B) Quantitative assessment of lentivirus vector transduction efficiency of mixed glial cells with lentivector diluted in 15% pluronic F127 gel.

the site of injection was evident. Immunofluorescent staining for GFP in this area revealed a large number of transduced cells (Fig. 5A), which were associated with a high level of Ox42 immunostaining for activated macrophages (Fig. 5B).

Immunostaining of serial sections of transduced tissue with GFP and GFAP antibodies revealed a large number of transduced astrocytes in areas that had been injected with viral vector alone (Fig. 6A and B) or vector complexed in pluronic F127 (Fig. 6C and D).

4. Discussion

Poloxamer based gels have previously been used to deliver adenoviral vectors to cells of the vasculature both in vitro and in vivo [8,9]. These vectors are based on a DNA virus and contain an outer protein coat, unlike lentiviral vectors, which are based on an RNA virus and contain an outer envelope derived from the plasma membrane of the cell the virus exits. Our in vitro results of 293T cell transduction with lentivirus vector diluted in PF 127 gel demonstrate that the gel does not damage the integrity of the vector or reduce its transduction efficiency. Previous reports have shown that poloxamer 407 gel increases the transfection efficiency of adenoviral vector delivery to vascular cells [9], suggesting slower kinetics in terms of vector release from the gel or the closer proximity of viral vector to the cell surface. We observed no increase in transduction efficiency of lentivirus vector delivery to both 293T cells or primary rat mixed glial cells, suggesting different kinetics for lentivirus vector release from PF127. The in vitro studies also highlighted the lack of toxicity associated with PF127 with no significant cell loss of either 293T or glial cells.

The glial cell culture contains a mixture of predominantly astrocytes but also a lower number of oligodendrocytes and fibroblasts. The presence of lentiviral vector in 15% pluronic gel did not appear to alter its cellular tropism with an increased number of transduced astrocytes at the highest input of vector (20 ng), lower concentrations of lentiviral vector with or without PF127 transduced a relatively similar number of astrocytes.

Delivery of lentivirus vector to the rat brain and spinal cord is achieved by stereotaxic injection. The thermoresponsive behaviour of PF127 allows the gel to be delivered in vivo over a short period of time before solidification. Using a localised pluronic gel based depot of

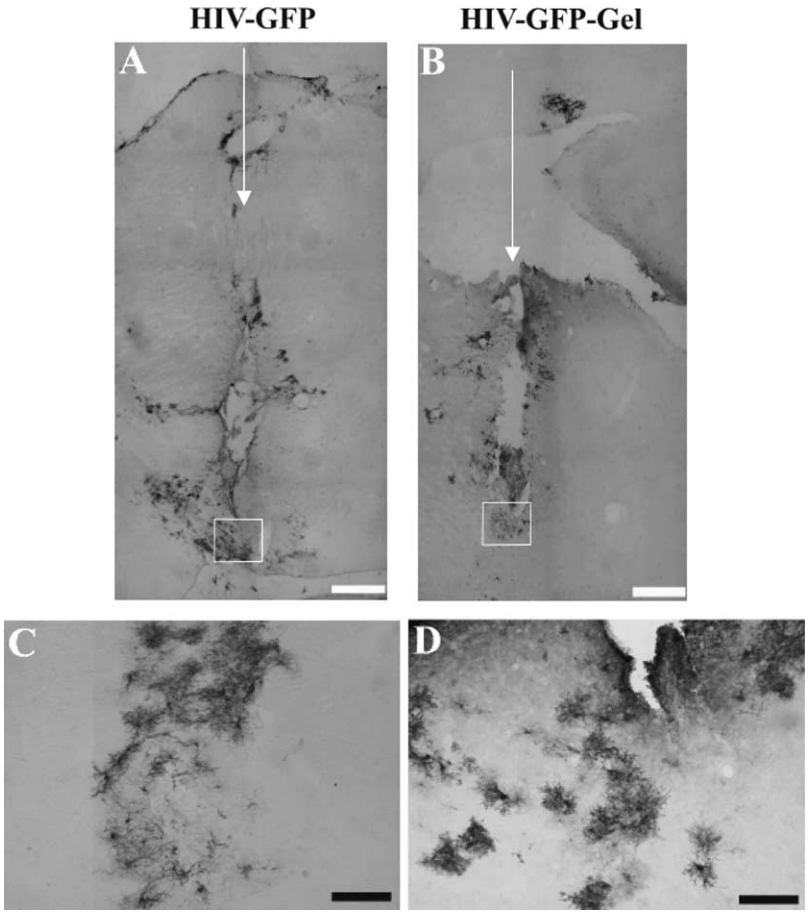

Fig. 4. (A and C) Immunostaining of GFP expression following stereotaxic delivery of HIV-1 lentiviral vector diluted in PBS +0.1% BSA, expressing GFP to the thalamus area of the rat brain at ×4 magnification (A) and ×20 magnification (C). (B and D) Immunostaining of GFP expression following stereotaxic delivery of HIV-1 lentiviral vector diluted in 15% pluronic F127 gel to the rat brain at ×4 magnification (B) and ×20 magnification (D).

viral vector release in the CNS would have application in brain injury or ischaemia and spinal cord trauma with the area of tissue damage capturing the semi solid gel. Delivery of adenoviral vector in poloxamer 407 gel to the vasculature has been via an endoluminal route with increased local transfection efficiency [9] and more recently Pluronic F127 containing adenoviral vector has been applied to the perivascular surface of the common carotid artery of the rat [22]. Our in vivo results now demonstrate that PF127 can be further applied to lentiviral vector delivery to the CNS by stereotaxtic injection. However delivery to the parenchyma close to the sub ventricular zone resulted in some tissue damage and an increase in activated macrophages, most probably due to the injection pressure of the gel. With sufficient release of the lentivirus vector in a localised area as demonstrated in Fig. 4, the application of

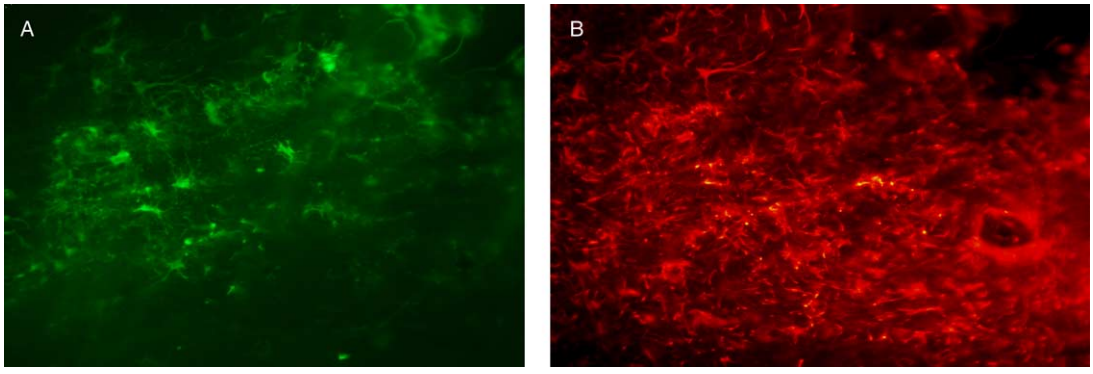

Fig. 5. Immunofluorescence detection of GFP protein expression in the brain following strereotaxic delivery by Lentiviral vector in pluronic F127 (Fig. 6A) and immunofluorescence detection of OX42 expression in the same area.

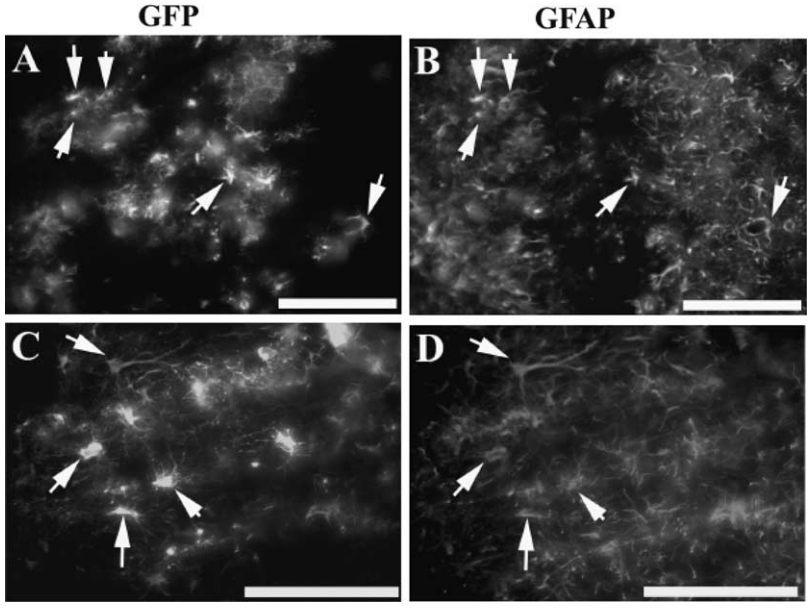

Fig. 6. Immunofluorescence detection of GFP and GFAP positive cells in serial sections of the thalamus region, injected with HIV-1 GFP lentiviral vector in PBS +0.1% BSA (A and B) and HIV-1 GFP lentiviral vector in 15% pluronic gel (C and D).

co-delivered PF127 and lentiviral vector will have most use in areas of CNS tissue loss or direct injection into a glial scar formed after brain or spinal cord injury [23]. Combined pluronic gel and lentiviral vector could be used to coat a lattice matrix such as polyglycolic acid, which has recently been used to promote axon regeneration in an experimental model of ischaemia [24]. Our present findings that PF127 can hold and deliver lentiviral vector both in vitro and in vivo, will allow further evaluation of modifications to PF127, which alter the gelation characteristics of the gel and may increase viral vector release kinetics. Such modifications of pluronics have included the addition of polyethylene glycol [14] and cellulose derivatives [15] to reduce the dissolution rate of drug from the gel. The addition of polyacrylic acid [16] or polycarbophil has increased the muco-adhesiveness of pluronics for improved nasal delivery of plasmid DNA [19]. Modification of the PHPMA hydrogel with an RGD peptide based on the adhesive region of fibronectin also promoted axonal outgrowth in the injured rat spinal cord [19] and implantation of Schwann cells into a into a poly (2-hydroxyethylmethacrylate) (poly HEMA) hydrogel has induced axonal regrowth in the lesioned optic tract of the rat [20]. A combination of a pluronic based gel containing lentiviral vectors expressing neurotrophic factors such as NT3 or BDNF with a PHPMA hydrogel or a biodegradeable scaffold based on poly-beta-hydroxybutyrate (PHB) [21] may contribute to axon regeneration and survival following CNS trauma.

In summary the data indicates that pluronic F127 gel can successfully contain a lentiviral vectors and allow delivery to cells in vitro without causing damage or loss of titre to the vector or toxicity to primary glial cultures. Stereotaxic delivery of vector and gel to the brain is possible but is associated with tissue damage and inflammation.

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