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Multifunctional, self-assembling anionic peptide-lipid nanocomplexes for targeted siRNA delivery

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ABSTRACT

Formulations of cationic liposomes and polymers readily self-assemble by electrostatic interactions with siRNA to form cationic nanoparticles which achieve efficient transfection and silencing *in vitro*. However, the utility of cationic formulations *in vivo* is limited due to rapid clearance from the circulation, due to their association with serum proteins, as well as systemic and cellular toxicity. These problems may be overcome with anionic formulations but they provide challenges of self-assembly and transfection efficiency. We have developed anionic, siRNA nanocomplexes utilizing anionic PEGylated liposomes and cationic targeting peptides that overcome these problems. Biophysical measurements indicated that at optimal ratios of components, anionic PEGylated nanocomplexes formed spherical particles and that, unlike cationic nanocomplexes, were resistant to aggregation in the presence of serum, and achieved significant gene silencing although their non-PEGylated anionic counterparts were less efficient. We have evaluated the utility of anionic nanoparticles for the treatment of neuronal diseases by administration to rat brains of siRNA to *BACE1*, a key enzyme involved in the formation of amyloid plaques. Silencing of *BACE1* was achieved *in vivo* following a single injection of anionic nanoparticles by convection enhanced delivery and specificity of RNA interference verified by 5′ RACE-PCR and Western blot analysis of protein.

1. Introduction

SiRNAs are double-stranded nucleic acids of 20–23 nucleotides which exploit endogenous enzymatic pathways found ubiquitously in nature to regulate gene expression by degradation of messenger RNA leading to gene silencing [1]. SiRNA can be delivered as a synthetic molecule to silence any specific gene and thus offer a wide range of therapeutic opportunities for the treatment of diseases by silencing genes involved in pathogenic pathways [2]. Numerous clinical trials of siRNA therapies are currently in progress for diseases in the eye, liver and lung as well as cancers [3-5]. Safe and efficient delivery of siRNA to affected tissues, however, remains a major technical barrier to the implementation of siRNA therapies [6].

SiRNAs are susceptible to degradation by RNases that are ubiquitous *in vivo* and, as they are anionic, hydrophilic molecules, are unable to cross the cell membrane efficiently [3]. Other problems include overcoming further intracellular barriers such as endosomal release, and to avoid the non-specific uptake by the reticuloendothelial system (RES) by its efficient delivery into target tissues. Nanocomplex formulations for siRNA delivery are therefore widely used and most of these are cationic as they enable self-assembly, cell binding and efficient transfection [4]. However, cationic formulations may be problematic for some applications *in vivo* as they interact with proteins in biological fluids and non-specifically with cells and form aggregates, leading to toxicity, poor biodistribution and immune responses [7,8]. These effects can be partially mitigated by PEG shielding of the surface charge [7] but anionic nanoparticle formulations offer another solution to these problems. Assembly between anionic polymers or lipids and nucleic acids can be achieved by including cationic bridging agents in the formulations such as Ca^{2+} cations [9-11], polymers and PEI [12,13], electrostatic coating [14] and protamine [15] which have

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enabled the *in vitro* and *in vivo* delivery of siRNA. These have offered promising evidence of efficacy and improved toxicity of anionic siRNA nanoparticles compared to cationic formulations.

We have previously described multifunctional Receptor-Targeted Nanocomplex (RTN) formulations comprising mixtures of cationic liposomes (L) and cationic targeting peptides (P) which self-assemble, electrostatically on mixing with plasmid DNA (D) or siRNA (R) and achieved efficient *in vitro* and *in vivo* gene delivery [16-21]. RTN formulations comprise a peptide which packages nucleic acids through a cationic K_{16} motif and receptor-targeting ligand for cell binding. Cationic LPR nanocomplexes containing siRNA were optimized with regard to their transfection efficiency and biophysical properties [19,21]. The optimal liposome component of RTNs for siRNA delivery *in vitro* was the cationic lipid formulation DOTMA/DOPE, which probably enhances transfection by destabilizing the endosomal bilayer allowing release of the siRNA to the cytoplasm before endosomal degradation can occur [19].

In this study we have developed anionic PEGylated RTNs for siRNA delivery, consisting of anionic liposome and a sixteen-lysine peptide with a targeting motif and assessed *in vitro* for their biophysical properties, cytotoxicity and receptor-mediated transfection efficiency. We have previously shown that anionic particles achieved more widespread dispersal in the brains of rats than cationic nanoparticles when delivered by convection-enhanced delivery (CED) directly into the corpus callosum or striatum due to their lower affinity for anionic cell surface glycoproteins [22,23] and have therefore explored the potential of anionic siRNA nanocomplexes for neurodegenerative diseases. Beta-site amyloid precursor protein cleaving enzyme 1 (*BACE1*), which encodes β-secretase, is expressed in neurons and cleaves the amyloid precursor protein (APP) to generate amyloid-beta (Aβ) peptides. This protein is over-expressed in the brains of patients with Alzheimer's disease (AD) and is proposed to lead to the production of amyloid plaques [24]. Aβ peptides play a crucial role in AD pathogenesis, and aggregates of these peptides initiate a cascade which ultimately leads to neurodegeneration [25]. *BACE1* is therefore a target for the treatment of AD but efficient delivery modalities are required that circumvent the blood brain barrier and transfect neurons throughout the brain. In this study we have optimized anionic nanocomplexes *in vitro* and then administered a formulation with *BACE1* siRNA into rat brain by CED and assessed target gene silencing as a therapeutic option for AD.

2. Materials and methods

2.1. Materials

1,2-dioleoyl-sn-glycero-3-phospho-(1'-rac-glycerol) (DOPG), 1,2-di-O-octadecenyl-3-trimethylammonium propane (DOTMA), 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE), 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000] (DPPE PEG2000), 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000] (DOPE PEG2000) and DOTMA/DOPE (1:1 molar ratio) were purchased from Avanti Polar Lipids (Alabaster, Alabama, USA). The structures of the lipids used are shown in Supplementary Table S1. Peptide Y (K_{16}GACYGLPHKFCG), Peptide ME27 (K_{16}RVRRGACRGDCLG) and non-targeting peptide ME72 (K_{16}RVRRGACRGECLG) were synthesized by ChinaPeptides (Shanghai, China), whereas non-targeting peptide K16 (KKKKKKKKKKKKKKKK) was synthesized by Alta Biosciences (Birmingham, UK). The siRNAs used for *in vitro* studies were: Silencer GAPDH siRNA, Silencer Firefly Luciferase (GL2+GL3) and Silencer Negative Control #1 siRNA all bought from Applied Biosystems (Warrington, UK). The siRNA for BACE1 *in vivo* studies was bought form Eurofins MWG Operon (Ebersberg, Germany) and the sequences were: BACE1 (sense) 5' GCUUUGUGGAGAUGGUGGAdTdT3' and BACE1 (antisense) 5' UCCACCAUCUCCACAAAGCdTdT 3'. Lipofectamine 2000 (L2K) was purchased from Invitrogen (Paisley, UK).

2.2. Liposome formulation

Lipids were dissolved in chloroform at 10 mg/ml and mixed, followed by production of a lipid film by slowly evaporating the chloroform. Lipids were rehydrated with sterile, distilled water whilst constantly being rotated overnight and then sonicated in a water bath to reduce their size. Anionic liposomes made were: DOPG:DOPE (L^A) at 1:1 molar ratio; DOPG:DOPE:DPPE PEG2000 ($L^{AP}1$) at a molar ratio of 47.5:47.5:5 mol%, respectively; DOPG:DOPE:DOPE PEG2000 ($L^{AP}2$) at a molar ratio of 47.5:47.5:5 mol%, respectively.

2.3. Nanoparticle formulation

Anionic nanocomplexes were prepared in water at different molar charge ratios of L to R, with the peptide P to R molar charge ratio constant at 3:1, by two methods. Method 1 (L:R:P): siRNA was first added to one of the liposomes L^A, $L^{AP}1$ or $L^{AP}2$ and incubated for 15 min at room temperature and then the peptide was added with rapid mixing and incubated at room temperature for a further 20 min; Method 2 (P:R:L): the peptide was added to the siRNA and incubated for 15 min at room temperature and then liposome was added with rapid mixing and incubated at room temperature for a further 20 min. For both methods of mixing the weight ratio of liposome to siRNA was 19:1 for L^A and 20:1 for $L^{AP}1$ and $L^{AP}2$. Cationic LPR (1:4:1 weight ratio of liposome:peptide:siRNA) formulations were made by first adding the peptide to the liposome DOTMA/DOPE followed by addition of the siRNA with rapid mixing and incubated for 30 min at room temperature to allow for complex formation. The nanocomplexes prepared were termed LPR (liposome DOTMA/DOPE), PRL^A or LRP^A (liposome L^A), $PRL^{AP}1$ (liposome $L^{AP}1$) and $PRL^{AP}2$ (liposome $L^{AP}2$).

2.4. In vitro transfections

Neuro-2A cells (ATCC, Teddington, UK) and Neuro-2A-Luc cells stably expressing luciferase [21] were maintained in Dulbecco's minimal essential medium (DMEM; Invitrogen, Paisley, UK) supplemented with 10% foetal calf serum, 1% non-essential amino acids, and 1% sodium pyruvate. The human airway epithelial cells 1HAEo− were provided by D. Gruenert, (San Francisco, CA, USA) and were cultured in Eagle's minimal essential medium (MEM) with (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) modification (Sigma–Aldrich, Poole, UK) supplemented with 10% foetal calf serum, 100 U/ml penicillin, and 100 mg/ml streptomycin, and 2 mol/l L-glutamine. All cells were maintained at 37 °C in a humidified atmosphere in 5% carbon dioxide.

Cells were seeded in 96-well plates at 2×10^4 per well 24 h prior to transfection. Following removal of growth medium, 200 μl of the complexes in OptiMEM containing 50 or 100 nM siRNA were added to the cells in replicates of six. Plates were centrifuged at 1500 rpm for 5 min (400 × g) and incubated for 4 h at 37 °C, then medium was replaced by the full growth medium and incubated for a further 24 h. In transfections where serum was present complexes made in OptiMEM were added to different amounts of complete media in order to obtain 5% and 10% serum-containing media. L2K was used as a positive control for siRNA transfections and the transfection procedures were performed in accordance with the manufacturer's instructions, formulated at a weight ratio of 4 (L2K): 1 (siRNA). Luciferase expression was then measured in cell extracts with a luciferase assay (Promega, Southampton, UK) in a FLUOstar Optima luminometer (BMG Labtech, Aylesbury, UK). The amount of protein present in each cell lysate was determined with the Bio-Rad protein assay reagent (Bio-Rad Laboratories, Hemel Hempstead, UK) in a FLUOstar Optima luminometer. Luciferase activity was expressed as relative light units per milligram of protein (RLU/mg). Each measurement was performed in groups of six and the mean determined. The GAPDH gene knockdown assays were conducted 48 h following transfection of Neuro-2A and 1HAEo-cells with RTNs containing Silencer GAPDH siRNA or Silencer Negative Control #1 according to the manufacturer's instructions of the KDalert GAPDH assay kit (Applied Biosystems, Warrington, UK). Experiments were repeated three times.

2.5. Particle size and charge measurements

Nanocomplexes were prepared as above and diluted with distilled water to a final volume of 1 ml and a concentration of 5 μg/ml siRNA. They were then analyzed for size and charge (ζ potential) using a Malvern Nano ZS (Malvern, UK). The data were then processed by software provided by the manufacturer, DTS version 5.03. All formulations had a polydispersity index (PDI) of less than 0.3. The values for each formulation are the means of three measurements and the experiments were repeated three times.

2.6. Turbidity assay

The absorbance of complexes in triplicates in the absence and presence of different serum concentrations (0–50% v/v) was measured at 500 nm on a FLUOstar Optima spectrophotometer as described previously with a corresponding amount of serum alone as a reference [23].

2.7. PicoGreen fluorescence quenching experiments

Briefly, 0.2 μg siRNA was mixed with PicoGreen reagent (1:150) (Invitrogen, Paisley, UK) at room temperature in TE buffer and the siRNA/PicoGreen mixture was then formulated into nanocomplexes with anionic liposome and cationic peptide into nanocomplexes as described above. Fluorescence was analyzed using a fluorescence plate reader, FLUOstar Optima (BMG Labtech, Aylesbury, UK). Heparin

dissociation assays were performed in triplicates for each formulation as described previously [21] and repeated three times.

2.8. Transmission electron microscopy (TEM)

For the electron microscopy investigations, the anionic nanoparticles prepared as described above were applied onto a 300-mesh copper grid coated with a Formvar/carbon support film (Agar Scientific, Stansted, UK) and processed as described previously [23].

2.9. Scanning electron microscopy (SEM)

Samples in 8% trehalose (Sigma–Aldrich, Poole, UK) were first freeze-dried and then adhered onto a SEM stub (Agar Scientific) using double-sided sticky carbon discs (Agar Scientific, Stansted, UK) and the excess sample was blown off with compressed air. Then the prepared stub was given a 10 nm gold coating in a Quorum Q150T Sputter Coater (Quorum Technologies, Lewes, UK) prior to viewing. Digital images were captured under a FEI Quanta 200F SEM (FEI Company, Eindhoven, Netherlands).

2.10. Cell proliferation assay

Cell viability of Neuro-2A cells was assessed in 96-well plates in replicates of six using the CellTiter 96 Aqueous One Solution Cell Proliferation Assay (Promega, Southampton, UK), as described previously [23].

2.11. Confocal microscopy

1.5×10^5 Neuro-2A cells were seeded onto poly-L-lysine coated slides (SLS, Dublin, Ireland). The following day they were transfected with Cy3-labelled GAPDH siRNA (100 pmol; final concentration of siRNA = 200 nM; Applied Biosystems, Warrington, UK) complexed with L2K at a 4:1 weight ratio or PRL formulations in triplicates. After 4 h incubation the slides were processed and visualized on a Carl Zeiss LSM710 laser scanning microscope system (Jena, Germany) at a magnification of x400 as described previously [21]. For the GAPDH silencing experiments the Neuro-2A cells were transfected in triplicates as above with GAPDH siRNA or irrelevant control siRNA complexed with different nanocomplexes. After 48 h incubation the slides were processed and visualized on a Carl Zeiss LSM710 laser scanning microscope system at a magnification of x400 as described previously [21].

2.12. In vivo CED procedures

All *in vivo* studies were performed in accordance with University of Bristol animal care policies and with the authority of appropriate UK Home Office licences. Adult male Wistar rats (Charles River, Margate, UK, 225–275g) were used for CED as described previously [23]. A total volume of 5 μl (5.6 μg of siRNA or 0.02 mg/kg) of anionic PEGylated nanoparticles in 5% dextrose was delivered to the striatum at an infusion rate of 2.5 μl/min. Rats were euthanized, and striata were placed in RNAlater (Invitrogen, Paisley, UK).

2.13. qRT-PCR

Rat brains were collected in RNAlater (Invitrogen, Paisley, UK) and total RNA was extracted from rat brain using the RNeasy kit according to the manufacturer's instructions (Qiagen, Crawley, UK). RNA was checked for integrity using the Agilent 2100 Bioanalyzer (Wokingham, UK). All RNA samples had an RNA integrity number (RIN) of more than 8 indicating high quality RNA. Prior to reverse transcription, each RNA sample underwent DNase treatment (Invitrogen, Paisley, UK) to eliminate any potential genomic DNA contamination. First-strand DNA was synthesized from 1 μg of DNase-treated RNA, using random hexamers and Superscript II reverse transcriptase (Invitrogen, Paisley, UK) in a 1 h reaction at 37 °C. Rat BACE1 and rat beta-actin were then quantified using Taqman primers and probes (Rn00569988_m1 and Rn00667869_m1, respectively; Life Technologies, Paisley, UK) and an ABI PRISM 7000 Sequence Detection System (Applied Biosystems, Warrington, UK). The qRT-PCR assay conditions were: stage 1, 50 °C for 2 min; stage 2, 95 °C for 10 min; stage 3, 95 °C for 15 s, then 60 °C for 1 min; repeated 40 times.

2.14. Western blot

Total protein was extracted from rat brain striatum in lysis buffer consisting of 75 mM Tris–HCl, 1% SDS and cocktail of protease inhibitor (Roche, West Sussex, UK) using Precellys Homogeniser (Stretton Scientific, Stretton, Derbyshire, UK). Protein was quantified by BCA protein assay kit (Thermo Fisher Scientific, Northumberland, UK). Forty micrograms of protein was loaded and separated using NuPAGE Pre-cast gels (10% Bis-Tris, Invitrogen, Paisley, UK) and then transferred electrophoretically to PVDF membrane (Invitrogen, Paisley, UK). The membrane was blocked in 10% skimmed milk in phosphate buffered saline buffer with 0.5% Tween-20 (PBS-T) at 4 °C overnight and then probed with the primary antibodies at room temperature for 1 h. After washing in PBS-T, membranes were incubated with HRP-conjugated anti-mouse or anti-rabbit IgG (Stratech Scientific, Suffolk, UK; 1:50,000) for 1 h at room temperature. Membranes were then rinsed in PBS-T thoroughly at room temperature before visualized by enhanced chemiluminescence detection kit (Bio-Rad, Hemel Hempstead, UK). The primary antibodies used in this study include rabbit anti-BACE1 polyclonal antibody (EE-17, 1:1000; Sigma–Aldrich, Poole, UK) and mouse anti β-tubulin monoclonal antibody (1:5000; Sigma–Aldrich, Poole, UK). Semi-quantification of the bands was performed by densitometry using the ImageJ software.

2.15. 5' rapid amplification of the 5' cDNA ends-PCR (5'RACE-PCR)

To confirm the BACE1 mRNA cleavage, following RNAi-mediated gene silencing with BACE1 siRNA, 5'-RACE PCR was performed following the manufacturer's instructions (GeneRacer™ Kit, Invitrogen) with some modifications. Briefly, total RNA from each sample was ligated to the GeneRacer RNA oligo linker without prior treatment. After Phenol/Chloroform precipitation, first-strand cDNA was reverse transcribed using the superscript™ III RT and a Gene-Specific Reverse Primer (Supplementary Table S2). 1 μL cDNA was amplified with GeneRacer™ 5' Primer and the gene specific reverse primer (Supplementary Table S2) as follows; 1 cycle of 95 °C for 2 min, then 15 touch-down cycles (repeating 3 cycles of 95 °C for 30 s, annealing from 67.5 °C to 61.5 °C with an increment of −1.5 °C for 30 s, and 72 °C for 20 s), and 10 cycles of 95 °C for 30 s, 60 °C for 30 s, 72 °C for 20 s, then 1 cycle of 72 °C for 10 min. From this product, 5 μL were used for nested PCR with a GeneRacer™ 5' Nested Primer and a gene-specific reverse nested primer (Supplementary Table S2) as follows; 1 cycle of 95 °C for 2 min, then 15 touch-down cycles (repeating 3 cycles of 95 °C for 30 s, annealing from 62.5 °C to 56.5 °C with an increment of −1.5 °C for 30 s, 72 °C for 20 s), and 10 cycles of 95 °C for 30 s, 60 °C for 20 s, 72 °C for 20 s, and then 1 cycle of 72 °C for 10 min. 5 μl of the nested PCR product was then re-amplified with an identical protocol of nested 5'-RACE PCR as described above. All PCR products were purified with MineElute® PCR Purification Kit (Qiagen) following the manufacturer's instructions.

2.16. Statistical analysis

Data presented in this study are expressed as the mean ± standard deviation of measurements performed in triplicates or replicates of six. They were then analyzed using a two-tailed, unpaired Student *t*-test or one way ANOVA and Bonferroni post-hoc analysis where applicable. Probability values $p < 0.05$ were marked with *, $p < 0.01$ were marked with ** and $p < 0.001$ were marked with ***.

3. Results

3.1. Development and biophysical characterization of anionic nanoparticles

LRP (Method 1) or PRL (Method 2) nanocomplexes were formulated at anionic liposome:siRNA (L:R) molar charge ratios from 5:1 to 1:1. LRP and PRL nanocomplexes were both strongly anionic when the L:R molar charge ratio was 3:1 or greater with no significant size or charge differences (Fig. 1S-A and S-B). PicoGreen fluorescence assays (Fig. 1S-C) showed that PRL formulations quenched approximately 50% more fluorescence than LRP formulations at all the ratios that produced anionic nanocomplexes (L:R ratios of 5:3, 4:3 and 3:3). Therefore, based on the efficiency of packaging and on our recent study where PDL nanocomplexes resulted in better DNA transfection efficiencies [23], the PRL method of mixing was used thereafter for all anionic nanocomplexes.

Anionic PEGylated nanocomplexes were then prepared with PEG2000 lipids incorporated at a 5% molar ratio in liposomes based on previous studies of extended circulation times and our own study [23,26]. Two different PEG lipids were used, DPPE-PEG2000 in $PRL^{AP}1$ and DOPE-PEG2000 in $PRL^{AP}2$. Anionic nanocomplex formulations were prepared at molar charge ratios of 5:3:1 and 4:3:1 and the zeta potentials of the PEGylated nanocomplexes at both ratios were shown to be anionic (−23 to −33 mV) with no charge differences between them (Fig. 1A). $PRL^{AP}2$ formulations were bigger than both $PRL^{AP}1$ and non-PEGylated PRL^{A} (Fig. 1A) while the charges of both PEGylated nanocomplexes were less anionic than non-PEGylated nanocomplexes (e.g. −25 mV for $PRL^{AP}1$ vs −40 mV for PRL^{A} at 4:3:1M molar charge ratio).

PicoGreen fluorescence quenching studies were performed to evaluate the effectiveness of packaging of siRNA within nanocomplexes in the presence or absence of 50% serum (Fig. 1B). The cationic LPR formulation provided the best packaging (>95%) but in the presence of serum 7.8-fold less siRNA was quenched. This contrasts to the anionic formulations where 80–91% packaging was

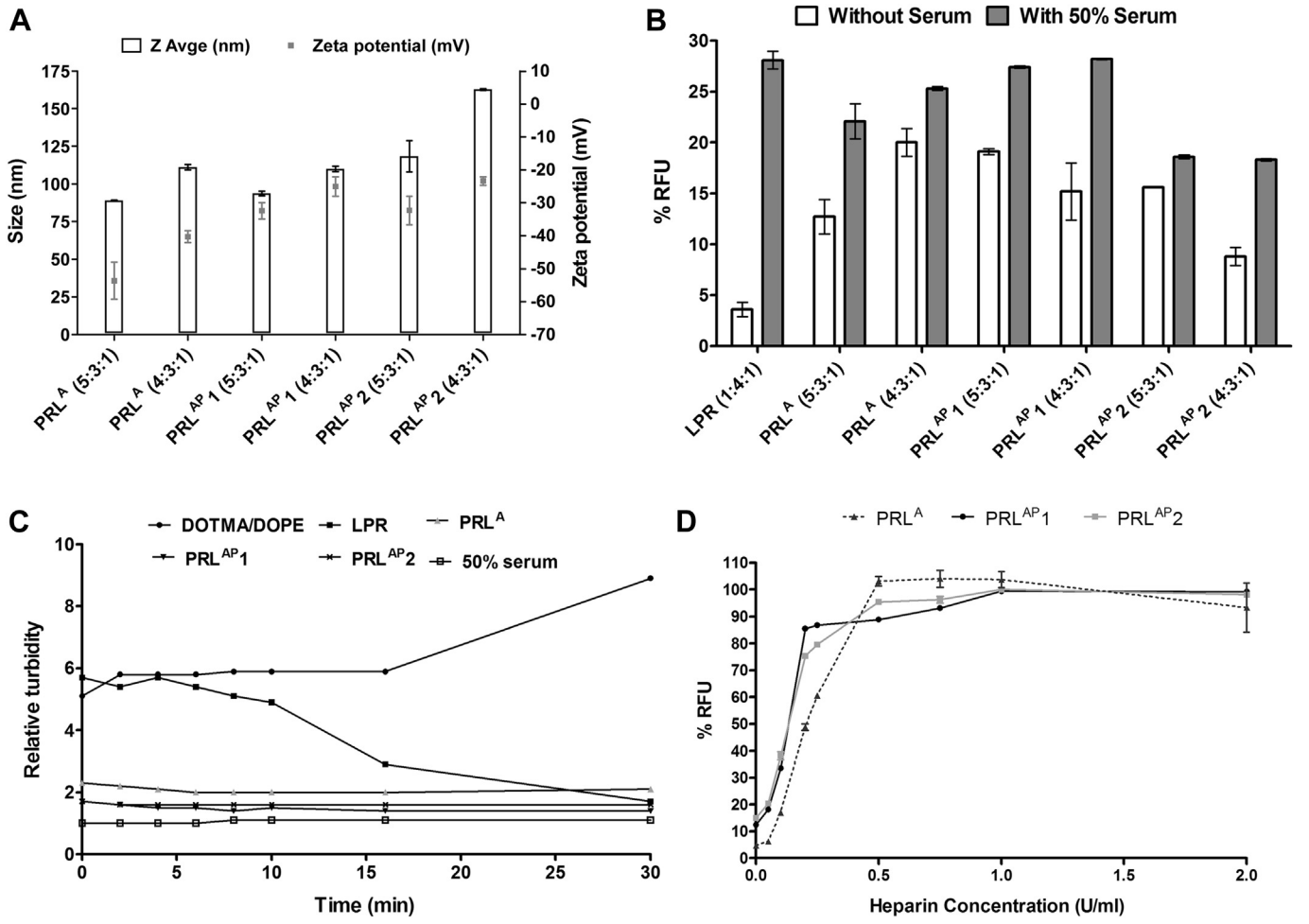

Fig. 1. Development of anionic nanoparticles and biophysical characterization. A) Anionic non-PEGylated PRL^{A} and PEGylated PRL^{AP}1 *and PRL*^{AP}2 nanoparticles were prepared at different molar charge ratios and their size and charge was measured by dynamic light scattering. B) Relative fluorescence unit (RFU) of the siRNA complexes as a percentage of signal from free siRNA in fluorescence quenching experiments in the presence or absence of 50% serum was measured for anionic nanoparticles that were made at different molar charge ratios and for cationic LPR nanoparticles. C) The effect of 50% serum concentration on the relative turbidity of cationic and anionic nanocomplexes over a 30 min incubation period. Cationic LPR nanocomplexes were made at 1:4:1 weight ratio, whereas the anionic nanocomplexes (PRL^{A}, PRL^{AP}1 and PRL^{AP}2) were all made at 4:3:1molar charge ratio. D) The dissociation properties of anionic PEGylated PRL^{AP}1 and PRL^{AP}2 and non-PEGylated PRL^{A} nanocomplexes at 4:3:1 M charge ratio were investigated. PicoGreen fluorescence of complexes, after incubation with heparin (0–2 U/mL), was expressed as a percentage of RFU relative to free siRNA.

achieved in the absence of serum, whereas when serum was added the quenching was only reduced by 1.2–2-fold.

We then compared the different nanocomplexes for their stability in 50% mouse serum by assessing aggregation with a turbidity assay. The turbidity of both, PEGylated and non-PEGylated, anionic nanocomplexes was less than half that of the cationic LPR formulation, with PRL^{A} showing slightly more turbidity than the PEGylated anionic nanoparticles (Fig. 1C). The ability of anionic nanocomplexes to package siRNA efficiently and to dissociate following heparin challenge in order to release intact siRNA was then assessed. PicoGreen-labelled siRNA was formulated into anionic, PEGylated and non-PEGylated nanocomplexes at 4:3:1 molar charge ratios. Packaging was inferred from quenching of fluorescence compared to free siRNA as 100%. Non-PEGylated PRL^{A} at 95% quenching, packaged the siRNA better than PEGylated PRL^{AP}1 and PRL^{AP}2 that both quenched siRNA fluorescence by approximately 85% (Fig. 1D). In addition, PRL^{A} was the least sensitive formulation to heparin as it achieved 50% dissociation at 0.21 U/mL heparin compared to PRL^{AP}1 and PRL^{AP}2 that both required 0.13 U/mL heparin. All formulations achieved 100% dissociation following heparin challenge at higher concentrations (0.5 U/mL for PRL^{A} and 1.0 U/mL for both the PRL^{AP}1 and PRL^{AP}2).

Anionic PRL nanoparticle formulations were further characterized by negative staining transmission electron microscopy (TEM) to determine shape and morphology. Most nanocomplexes were spheres but with some rods, while PRL^{AP}2 also showed some pleiomorphic structures (Fig. 2). The majority of the spherical particles for each formulation were in the range determined by particle size analysis with no obvious differences between formulations. SEM showed that anionic nanoparticles were spherical and immobilized in matrices of trehalose.

3.2. Enhanced targeted silencing and viability of cells transfected with anionic PRLs

PEGylated (PRL^{AP}1 and PRL^{AP}2) and non-PEGylated (PRL^{A}) formulations at 4:3:1 molar charge ratios were compared for silencing of luciferase in transfections of Neuro-2A-Luc cells (Fig. 3A). PRL^{AP}1 was significantly better by approximately 2.1–fold and 1.8-fold ($p < 0.01$ in both cases), than PRL^{AP}2 and PRL^{A} at 100 nM, respectively and was only inferior to L2K at 50 nM ($p < 0.05$). The same formulations were compared for silencing of GAPDH enzymatic activity in 1HAEo-cells (Fig. 3B). Again PRL^{AP}1 was significantly better by more than 2–fold ($p < 0.001$ in both cases), than PRL^{AP}2

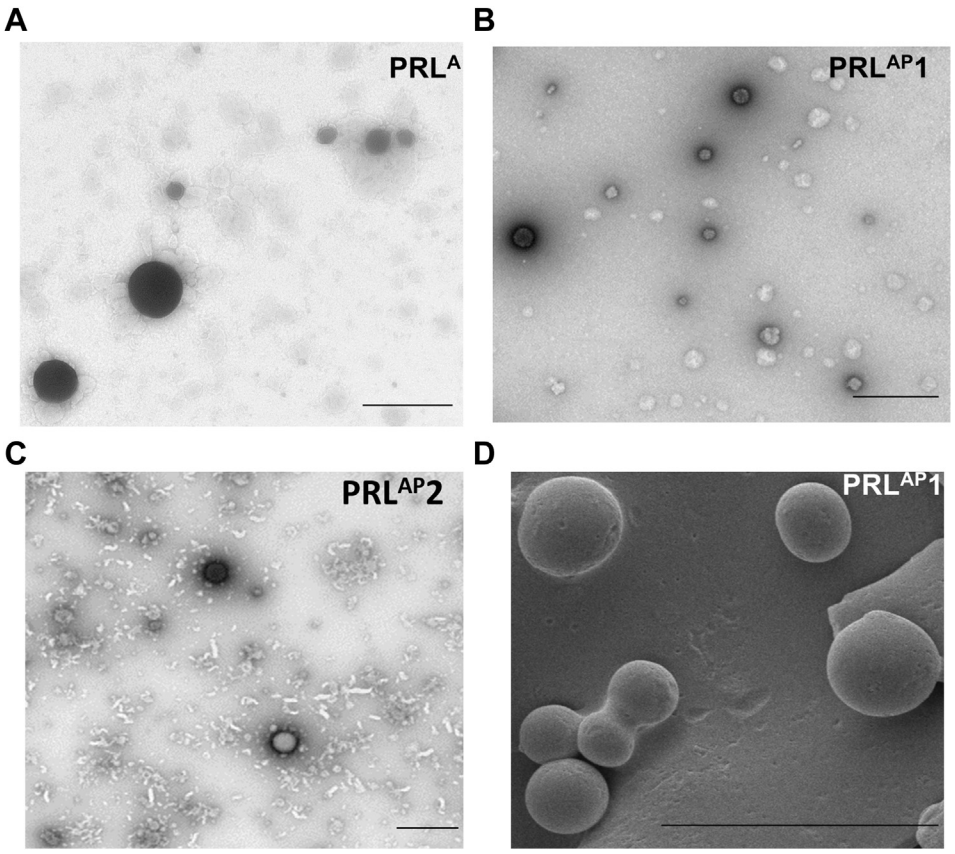

Fig. 2. Electron microscopy of nanocomplexes. Negative staining transmission electron microscopy was used to visualize A) PRL^{A}, B) $PRL^{AP}1$, and C) $PRL^{AP}2$ nanoparticles. Scale bar = 500 nm. D) Scanning electron microscopy was used to visualize $PRL^{AP}1$ nanoparticles. Scale bar = 4 μm.

and PRL^{A} at 100 nM, respectively, with no significant differences compared to L2K at 50 nM and 100 nM. In addition, when we compared $PRL^{AP}1$ formulations with targeting peptide Y or targeting peptide ME27 to non-targeting nanocomplexes, we achieved luciferase silencing in Neuro-2A-Luc cells only with the targeted nanoparticles (Fig. 2S).

We then assessed the ability of our anionic formulations to achieve gene silencing in the presence of serum in transfections of Neuro-2A-Luc cells. At both 10% (Fig. 3C) and 5% serum (Fig. 3D) $PRL^{AP}1$ resulted in 60% silencing, which was significantly better than either $PRL^{AP}2$ ($p < 0.05$) or PRL^{A} ($p < 0.001$ and $p < 0.01$ for 10% and 5% serum, respectively). L2K resulted in more than 85% silencing at both 5% and 10% serum, which was significantly better than $PRL^{AP}1$ only at 10% serum ($p < 0.01$).

Anionic PRL^{A}, $PRL^{AP}1$ and $PRL^{AP}2$ formulations were also compared for cytotoxicity using two different siRNA concentrations. MTS toxicity assays of transfected Neuro-2A cells (Fig. 3E) showed that all three anionic formulations resulted in minimal cytotoxicity with no differences between them.

3.3. Internalization of the siRNA complexes and silencing effect

Cellular uptake and distribution of siRNA delivered by anionic nanocomplexes was studied in Neuro-2A cells transfected for 4 h with formulations containing Cy3-labelled GAPDH siRNA. The cells were stained with phalloidin for the cytoplasm and DAPI for the nucleus, then assessed by confocal microscopy (Fig. 3S). Fluorescent anionic PEGylated and non-PEGylated nanoparticles appeared inside the cytoplasm at 4 h with no fluorescent siRNA observed in the nucleus. These data are in agreement with our previous studies where we had shown intracellular detection of cationic nanocomplexes at 4 h [19,21].

In silencing experiments, Neuro-2A cells were transfected with GAPDH siRNA and irrelevant control siRNA and analyzed by confocal microscopy with an anti-GAPDH antibody (Fig. 4). Silencing of GAPDH expression in Neuro-2A cells was evident following transfection with L2K, PRL^{A}, and $PRL^{AP}2$ but was even more pronounced with $PRL^{AP}1$ nanocomplexes, therefore providing further evidence for the silencing potency of the anionic PEGylated formulations.

3.4. In vivo administration by CED of anionic PRLs demonstrated silencing of BACE1

We then evaluated the *in vivo* knockdown of *BACE1*. $PRL^{AP}1$ nanoparticles incorporating either *BACE1* siRNA or irrelevant control siRNA were administered by CED into rat striata and *BACE1* expression was examined 48 h later. A significant reduction (approximately 60%) in *BACE1* mRNA was observed (Fig. 5A) between the *BACE1*-treated group and the control groups ($p < 0.01$ compared to the irrelevant control group and $p < 0.001$ compared to the untreated control group). Western blotting analysis revealed that BACE1 protein levels were also suppressed by ~30% compared to both controls (Fig. 5B). 5′ RACE-PCR analysis (Fig. 5C) detected the predicted 240 bp cleavage product of mRNA only in samples from BACE1 siRNA-treated rat brains and not in those receiving irrelevant siRNA control or in untreated rats. Thus, the reduction in *BACE1* expression demonstrated by mRNA quantification, mRNA cleavage analysis and the protein quantification support an RNAi-mediated mechanism of gene silencing.

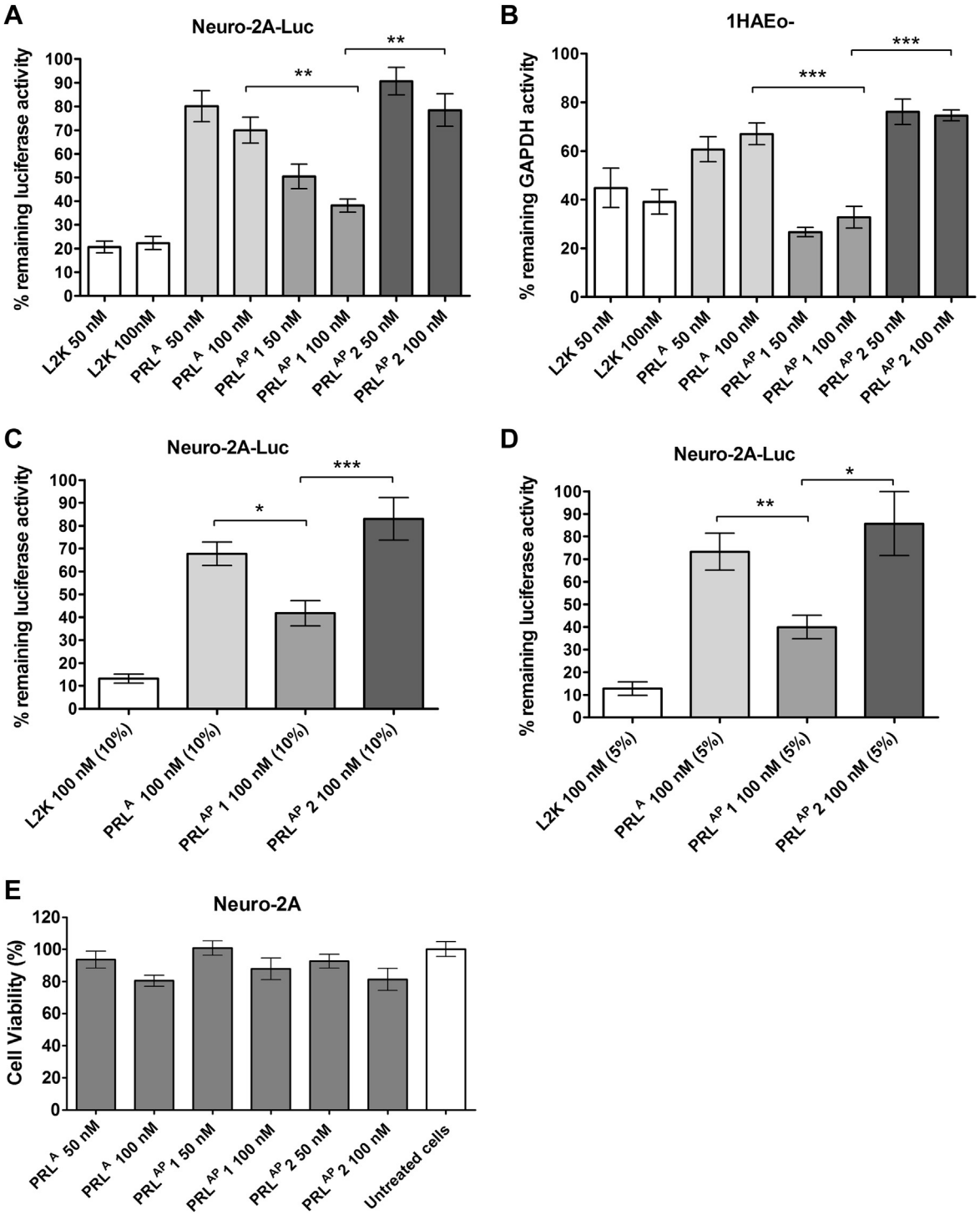

Fig. 3. SiRNA knockdown in the presence or absence of serum and cell viability. A) PRL^{A}, $PRL^{AP}1$, $PRL^{AP}2$ at 4:3:1 molar charge ratio using siRNA targeting luciferase in Neuro-2A-Luc cells at two concentrations, 50 and 100 nM. 24 h later luciferase assays were performed. B) PRL^{A}, $PRL^{AP}1$, $PRL^{AP}2$ at 4:3:1 molar charge ratio using siRNA targeting GAPDH in 1HAEo- cells at two concentrations, 50 and 100 nM. 48 h later GAPDH assays were performed. C) PRL^{A}, $PRL^{AP}1$, $PRL^{AP}2$ at 4:3:1 molar charge ratio using siRNA targeting luciferase in Neuro-2A-Luc cells at 100 nM were investigated for their ability to perform silencing in the presence of 10% serum. 24 h later luciferase assays were performed. D) PRL^{A}, $PRL^{AP}1$, $PRL^{AP}2$ at 4:3:1 molar charge ratio using siRNA targeting luciferase in Neuro-2A-Luc cells at 100 nM were investigated for their ability to perform silencing in the presence of 5% serum. 24 h later luciferase assays were performed. All results shown (A–D) have been normalized with values for the same formulations using irrelevant control siRNA which were set at 100%. L2K/siRNA nanocomplexes were used as a positive control in all *in vitro* silencing experiments. E) Viability of Neuro-2A-Luc cells following transfection for 24 h with PRL^{A}, $PRL^{AP}1$, $PRL^{AP}2$ at 50 and 100 nM (all siRNAs were targeting luciferase). Viability values were normalized to the untransfected control cells. All transfections were performed in groups of six and mean values were calculated. Asterisks indicate comparisons of specific formulations with statistical significance (*, $p < 0.05$ and ***, $p < 0.001$).

4. Discussion

Anionic nanocomplexes are attracting increasing scientific attention as an approach to circumvent problems associated with the *in vivo* use of cationic nanoparticles such as poor tissue specificity, toxicity and rapid clearance from the circulation. Anionic formulations for DNA delivery have shown evidence of improved biodistribution, efficacy and reduced toxicity compared to cationic

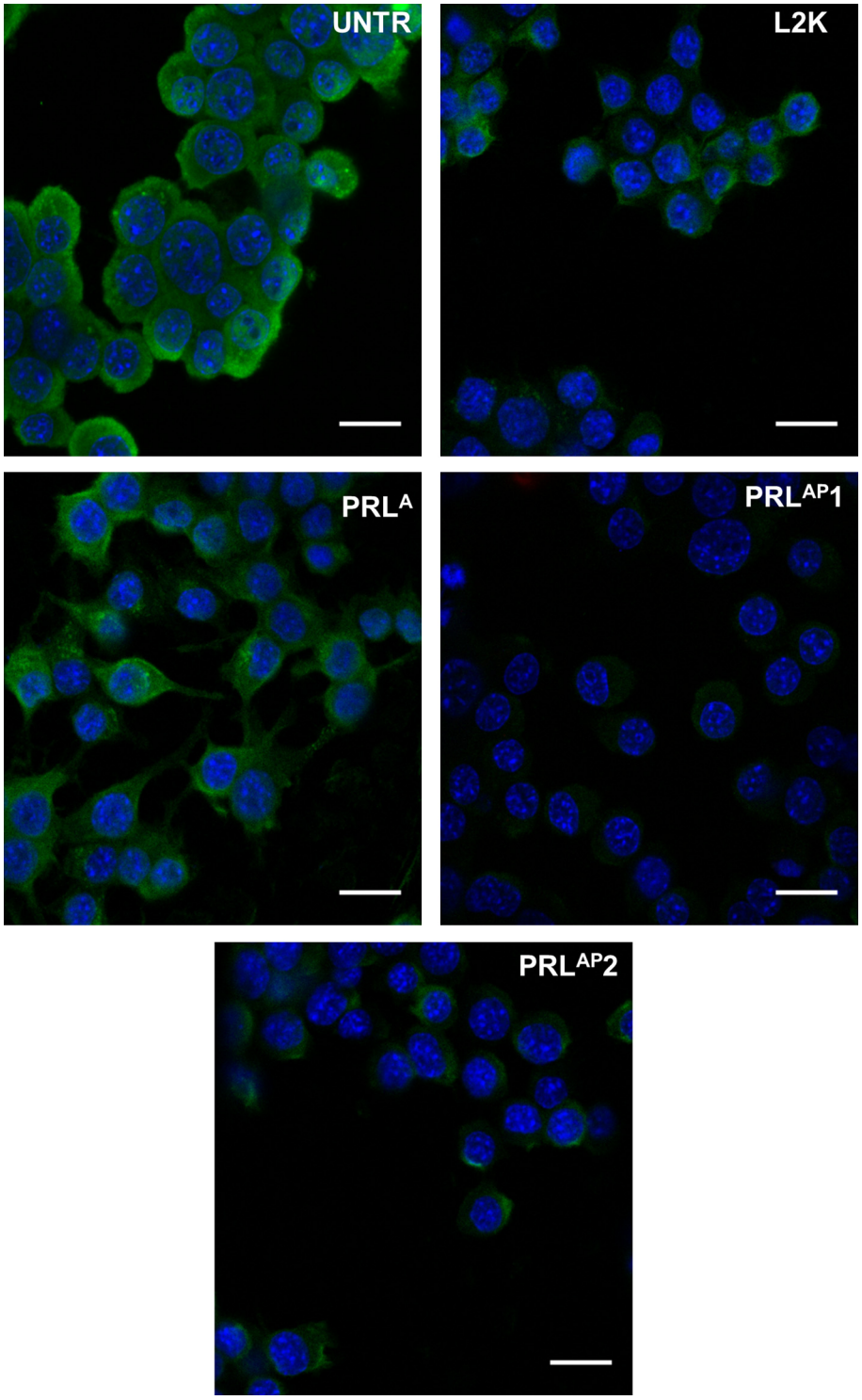

Fig. 4. SiRNA silencing of GAPDH gene expression. GAPDH siRNA and irrelevant control siRNA transfected into Neuro-2A cells, and analyzed by fluorescence microscopy with an anti-GAPDH antibody. Blue: DAPI for the nucleus Green: fluorescein-labelled antibody to GAPDH. SiRNA silencing of GAPDH expression in Neuro-2A cells is demonstrated following transfection with L2K, PRL^{A}, $PRL^{AP}1$ and $PRL^{AP}2$ complexes with siRNA targeting GAPDH. UNTR = untreated cells. Scale bar = 20 μm.

formulations following systemic administration for tumour targeting [26-29]. Anionic formulations have also been used for siRNA silencing with similarly promising results *in vitro* [9,10,30] and *in vivo* [14] including tumour targeting [11-13,15]. Hence, there is a need for additional research to optimize the design, formulation and applications of anionic nanocomplexes.

We are developing anionic, targeted formulations comprising a mixture of peptide ligands and anionic, PEGylated liposomes that self-assemble into anionic nanocomplexes with DNA or siRNA at optimized ratios of components and order of mixing. Our first such formulation with a DNA cargo displayed far superior distribution in rat brain than homologous cationic

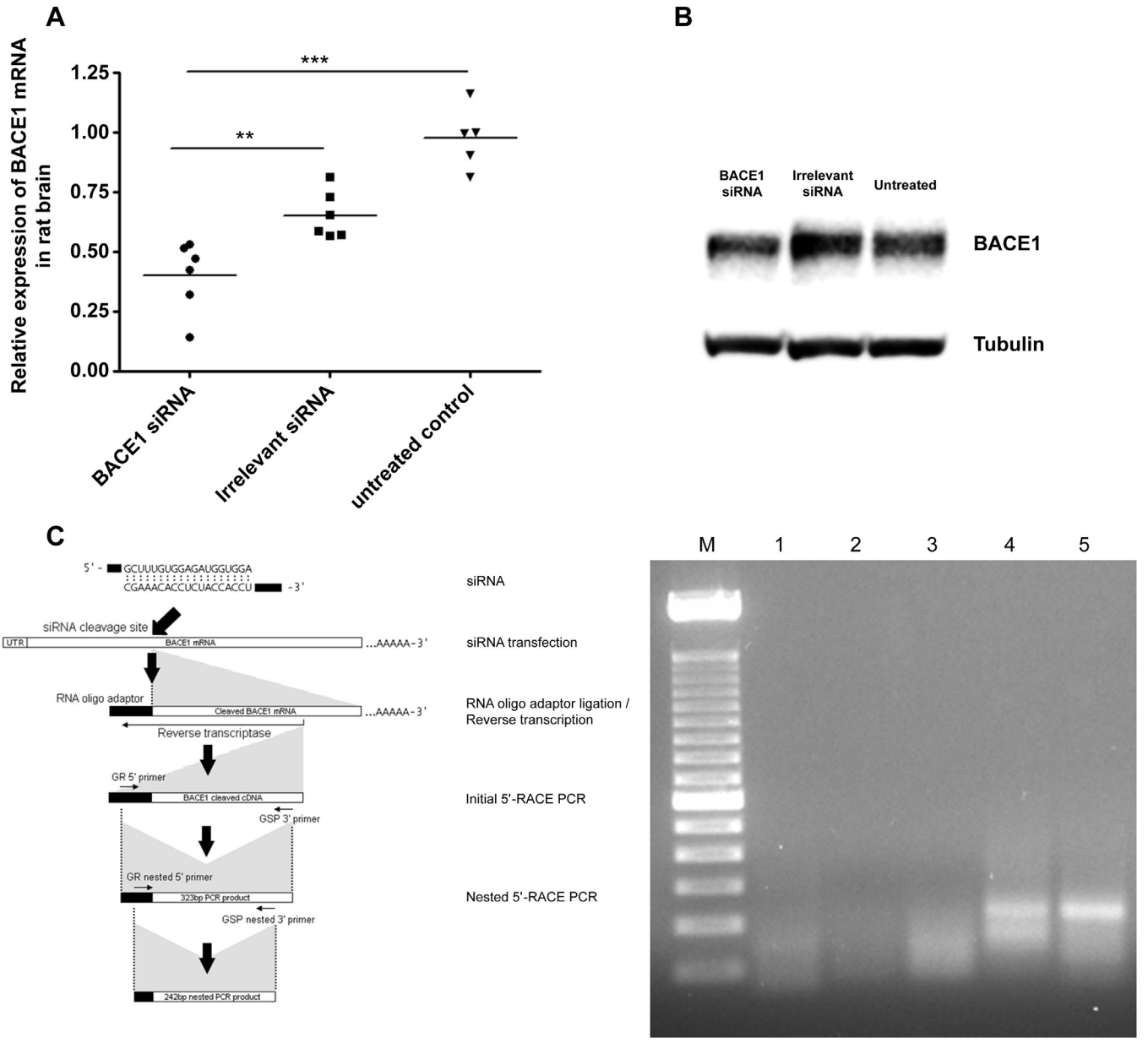

Fig. 5. *In vivo* silencing of BACE1 following CED administration of PEGylated nanoparticles into rat striatum. A) Anionic PEGylated PRL^{AP}1 nanoparticles with BACE1 siRNA or irrelevant control siRNA were administered by CED in the striatum of rats and 48 h post administration tissues were removed for qRT-PCR analysis of siRNA-induced silencing of *BACE1* gene. Values are the means of 6 animals ± standard deviation ($n = 5$ for the Untreated control animals) with one way ANOVA and Bonferroni post-hoc analysis performed to calculate significant differences (*, $p < 0.05$; ***, $p < 0.001$). B) Western blot analysis of BACE1 protein in rat striatum following CED administration of anionic PEGylated PRL^{AP}1 with BACE1 siRNA or irrelevant control siRNA, 48 h post-administration. Silencing was calculated with densitometric analysis using tubulin as loading control. C) SiRNA specific cleavage following CED administration of PRL^{AP}1 nanoparticles. A schematic diagram of the 5′ RACE-PCR method for *BACE1* mRNA cleavage is provided. Cleaved *BACE1* mRNA resulted in the 242 bp nested PCR products which are indicative of siRNA-specific cleavage. The actual gel image of the 5′ RACE-PCR products is shown. RNA was extracted from rat brains of mice which were either treated with BACE1 siRNA or irrelevant control siRNA encapsulated in PRL^{AP}1 nanoparticles or from brains of untreated control rats and was then subjected to 5′ RACE-PCR. M: 100 bp DNA ladder; Lane 1: Untreated rat; Lanes 2–3: irrelevant control siRNA treated rats; Lanes 4–5: BACE1 siRNA treated rats.

nanocomplexes following CED administration and increased transfection efficiencies *in vitro* [23]. Here we have focused on developing anionic nanoparticles for siRNA delivery and investigated the effects of PEGylated formulations. We relate the biophysical properties of these nanocomplexes to their silencing efficiencies *in vitro* and we report the effects of administration of these siRNA anionic nanocomplexes *in vivo*, in rat brain. We used a targeting peptide in the formulation that targets multiple cells with high transfection efficiency and was shown previously to be effective in the brain for targeted DNA delivery [19,21,23].

We first optimized methods of mixing and molar charge ratios, then structural and functional studies were performed to compare the PEGylated formulations with non-PEGylated anionic and cationic formulations. The anionic PEGylated nanoparticles of ~110 nm were similar (PRL^{AP}1) or slightly larger (PRL^{AP}2) in diameter than non-PEGylated anionic formulations (PRL^{A}), whereas their charge was less anionic due probably to PEG shielding [31]. TEM analysis of anionic nanoparticles showed that there were no major differences in morphology between the different PEGylated and non-PEGylated anionic formulations or cationic LPR nanocomplexes reported previously [21].

SiRNA packaging was less efficient in anionic formulations than in cationic LPR possibly because the two cationic moieties (liposome and peptide) in LPR exert more force to siRNA than the one cationic moiety (peptide) in the anionic formulations. In addition, PEGylation in PRL^{AP}1 and PRL^{AP}2 resulted in more efficient packaging of siRNA than the non-PEGylated PRL^{A}. We then tested the nanoparticle stability in turbidity assays using 50% mouse serum to better reflect *in vivo* conditions [32]. PEGylated and non-PEGylated PRL^{A} formulations were far more stable in serum than cationic nanocomplexes, which aggregated due to their interactions with anionic serum proteins as reported previously for cationic liposomes [33]. In addition, fluorescently labelled siRNA in cationic nanocomplexes was quenched 8-fold less in serum than in non-serum conditions, whereas the quenching of anionic nanoparticles was reduced less than two fold in serum compared to serum-free conditions. Aggregation of cationic nanocomplexes leads to reduced bioavailability and binding to cells, increased toxicity and clearance *in vivo* by the RES [32] and so the improved behaviour of anionic nanocomplexes in serum suggests that their bioavailability and activity *in vivo* should be enhanced.

We next assessed the stability of nanocomplexes to anionic challenge as an indication of their potential to dissociate and release siRNA in the cytoplasm. While extracellular stability is an essential requirement for an effective siRNA delivery formulation, effective transfection and silencing is also influenced by the ease of release of the siRNA within the cell following internalization [21]. PEGylated anionic nanocomplexes dissociated more readily than non-PEGylated formulations which required approximately 60% more heparin to achieve 50% dissociation. The different sensitivities of PEGylated and non-PEGylated anionic nanocomplexes to heparin, may be explained by the greater anionic charge of the latter.

We then compared the silencing efficiencies of the anionic formulations using two different cell lines, Neuro-2A-Luc and 1HAEo- in 5% and 10% serum or serum-free conditions. PRL^{AP}1—mediated silencing of 60–80% that was significantly better than PRL^{AP}2 and non-PEGylated PRL^{A} for both luciferase and the endogenous GAPDH gene in both, serum-containing or serum-free conditions PRL^{AP}1 and similar to levels achieved with L2K. Both cationic and anionic formulations prepared with two different targeting peptides, peptide Y and peptide ME27 [18,19,23,34], showed the necessity for the peptide targeting ligand where formulations with control, non-targeting peptides were inactive. Furthermore, confocal studies of silencing endogenous GAPDH also demonstrated the potential of the PEGylated anionic nanocomplexes for silencing. Toxicity assays showed that both PEGylated and non-PEGylated anionic formulations were not cytotoxic as also reported previously with other anionic formulations [14,23,35,36].

In relating differences in silencing efficiency to biophysical properties, PRL^{A} possessed the highest anionic charge and was the least efficient formulation for siRNA release and serum aggregation. The high anionic surface charge would reduce cell surface interactions and endocytosis and this, combined with its worse siRNA release kinetics helps to explain the poor silencing efficiency compared to PRL^{AP}1 which displayed better silencing efficiency associated with improved dynamic properties of the nanocomplexes in that it packaged and released siRNA effectively, aggregated less and had a lower anionic surface charge than the non-PEGylated formulation. Of the two PEGylated formulations, PRL^{AP}2 was slightly bigger than PRL^{AP}1 but otherwise presented with similar biophysical properties of charge and morphology and same properties for packaging, dissociation and serum aggregation. Therefore, the difference in silencing between PRL^{AP}1 and PRL^{AP}2 can only be explained by the fact that the PEGylated lipid moiety in PRL^{AP}1 is a C16 saturated acyl chain while in PRL^{AP}2 it is a C18 unsaturated acyl chain. The effectiveness of lipids with shorter alkyl tails in siRNA silencing might be explained by the fact that the shorter length of the alkyl chain is associated with greater alkyl tail flexibility and a lower phase transition temperature from crystalline to fluid phases of the bilayer in vesicles [37] and so may help to confer the greater gene silencing efficiency by promoting endosomal release of the siRNA.

We then performed *in vivo* experiments where we administered by CED nanoparticles for *BACE1* silencing. BACE1 is the rate-limiting enzyme in the production of Aβ peptides which are landmarks in AD pathophysiology [24]. Both the expression and enzymatic activity of BACE1 are increased in brains of AD patients [38], whereas in *BACE1* knockout mice there is no generation of Aβ peptides [39]. BACE1 is ubiquitously expressed in the brain, including high levels of expression in the striatum, with higher expression levels observed in neurons compared to other cell types [38]. BACE1 is therefore a main therapeutic target for AD. PRL^{AP}1 resulted in about a 60% reduction in *BACE1* mRNA compared to the untreated group, which was similar to a study using systemic injection and targeted exosomes [40], and 30% protein silencing. The group treated with the irrelevant control siRNA, a group not included in the exosome study [40], resulted in about 30% mRNA knockdown but this was unspecific as it did not show any protein silencing and importantly the 5'RACE analysis clearly confirmed that only PRL^{AP}1-BACE1 siRNA containing nanoparticles silenced *BACE1* through RNAi. BACE1 is also expressed by other cell types at lower levels e.g. astrocytes and microglia [41] and all three cell types (neurons, astrocytes and microglia) are present in the striatum [42]. Injury that may be caused by the implantation of the catheter during CED either directly attract microglia or might activate astrocytes [43,44] which in turn might mediate rapid microglial response through signalling [45,46]. Therefore, more cell types are present and thus the BACE1 levels might be changed. Non-specific silencing might be the result of this change in the cell population harvested and processed for mRNA isolation. It was reported that, as long as BACE1 protein was even reduced partially, Aβ levels may be decreased and that lowering the Aβ peptide generation by 20–30% is sufficient to retard the pathological development and defer the inception of symptomatic AD [38,47]. Hence, it is not necessary to entirely inhibit Aβ peptide generation to treat AD. Therefore, based on the present knowledge it is plausible that partial lowering of *BACE1* expression at the levels achieved here may prove beneficial to AD sufferers. Further studies using multiple, repeated siRNA dosing and investigating the duration of the silencing effect may achieve higher levels of silencing for a sustained period. Ideally, nanoparticles would be administered through the bloodstream and bypass the blood-brain barrier by taking advantage of endogenous receptors but this remains an obstinate challenge to the field and so, for now, CED provides an effective method of direct administration.

5. Conclusions

In this study we describe an anionic modular PEGylated nanocomplex and demonstrated enhanced silencing efficiency of different genes. The silencing efficiency of PRL^{AP}1 was comparable with that of the commercial transfection agent L2K, but without the significant cytotoxicity which is associated with the latter. Distribution of nanoparticles in the brain, administered by direct delivery, resulted in BACE-1 gene silencing demonstrating the potential to advance the field of siRNA therapeutics for several disorders.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.biomaterials.2014.06.003.

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