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

Aristides D. Tagalakis a, *, Do Hyang D. Lee a, Alison S. Bienemann b, Haiyan Zhou a, Mustafa M. Munye b, Luisa Saraiva a, David McCarthy c, Zixiu Du a, Conrad A. Vink a, Ruhina Maeshima a, Edward A. White b, Kenth Gustafsson c, Stephen L. Hart a

a Molecular Immunology Unit, UCL Institute of Child Health, 30 Guilford Street, London, WC1N 1EH, UK
b Functional Neurosurgery Research Group, School of Clinical Sciences, AMBI Labs, University of Bristol, Southmead Hospital, Bristol, BS10 5NB, UK
c UCL School of Pharmacy, 29-39 Brunswick Square, London, WC1N 1AX, UK

* Corresponding author. Tel.: +44 2079052817; fax: +44 2079052810.
E-mail addresses: a.tagalakis@ucl.ac.uk, a.tagalakis@ich.ucl.ac.uk
(A.D. Tagalakis).

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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 S’ RACE-PCR and Western blot analysis of protein.

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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++, cations [9-11], polymers and PEI [12,13], electrostatic coating [14] and protamine [15] which have
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 catonic lipid formulation DOTMA/DOP, 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 shown previously 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 siRNA to the brain cells than cationic nanoparticles when delivered by convection-enhanced delivery (CED) directly into the corpus callosum or striatum due to their lower affinity for anionic siRNA to the cytoplasm before endosomal degradation can occur [19].

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 1HEAO– were provided by D. Gruenert, (San Francisco, CA, USA) and were cultured in high Eagle’s minimal essential medium (MEM) with (4-2-hydroxyethyl)-1-piperazineethanesulfonic acid (Sigma–Aldrich, Poole, UK) supplemented with 10% foetal calf serum, 100 U/ml penicillin, and 100 mg/ml streptomycin, and 2 mol/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 cells in serum-containing media at a molar charge ratio of liposome to siRNA of 19:1 for Lube and 20:1 for Lube and similarly for Lube. Cationic LPR (1:4) weight ratio of siRNA:peptide:siRNA formulations were made by first adding the peptide to the liposome DOTMA/DOP followed by addition of the siRNA with rapid mixing and incubated for 30 min at room temperature to allow for complex formation. The nanocomplexes were prepared as described 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 absorbance of complexes in triplicates in the absence and presence of 50 or 100 μg/ml of protein (BSA or BSA) was measured at 562 nm using a spectrophotometer (Ultrospec 3000, Pharmacia, Uppsala, Sweden).

2. Materials and methods
2.1. Materials
1,2-dioleoyl-sn-glycerol-3-phosphate (DLDPG), 1,2-di-O-octanoyl-sn-glycerol-3-phosphoethanolamine (DOPE), 1,2-dipalmitoyl-snglycerol-3-phosphoethanolamine (DPPE), 1,2-dioleoyl-sn-glycerol-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000] (DPPE PEG2000), 1,2-dioleoyl-sn-glycerol-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000] (DPPE PEG2000), and DOTMA/DOP (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 (KvG4CYP1HFCG) was synthesized by Sino Peptide (Shanghai, China), whereas non-targeting peptide K16 (KKKKKKKKKKKKKKKKKKKKKKKKKKKKKKKKKKKKKK) was synthesized by Asia Biosciences (Birmingham, UK). The siRNAs used for in vitro studies were: Silencer GAPDH siRNA, Silencer Firefly Lucerase (GL2–GL3) and Silencer Negative Control #1 siRNA all bought from Applied Biosystems (Warrington, UK). The siRNA for BACE1 in vivo studies was bought from Eurofins MWG Operon (Ebersberg, Germany) and the sequence was: BACE1 (sense) 5’ GCUUUUGUGAGAAGGGUGGAdTdT3’ and BACE1 (antisense) 5’ UCCACCAUCCACAAAGGCdT3’. 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 redyeded with sterile, distilled water whilst constantly being rotated overnight and then sonicated in a water bath to reduce their size. Anionic liposomes were made: DOPC/DOPG (L1) at 1:1 molar ratio; DOPC/DOPG/DPPE PEG2000 (L2) at a molar ratio of 47:5:47:5:5 mol%, respectively; DOPC/DOPG/DPPE PEG2000 (L2) at a molar ratio of 47:5:47:5 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:P:R): siRNA was first added to one of the liposomes L1, L2 or L2 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:L:R): 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 L1 and 20:1 for L2 and L2. Cationic LPR (1:4) weight ratio of liposome:peptide:siRNA formulations were made by first adding the peptide to the liposome DOTMA/DOP followed by addition of the siRNA with rapid mixing and incubated for 30 min at room temperature to allow for complex formation. The nanocomplexes were prepared as described 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 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 (1:1500) (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 fluorescein plate reader, FLUOstar Optima (BMG Labtech, Aylesbury, UK). Heparin

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].
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% trolley (Sigma–Aldrich, Poole, UK) were first freeze-dried and then fractured. The Cverted 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, Southhampton, UK), as described previously [23].

2.11. Confocal microscopy

1.5 × 10⁶ 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 Biosysytems, Warrington, UK) complexed with Li2K at a 4:1 weight ratio or PRL formulations in triplicates. After 4 h incubation the slides were processed and visualised on a Carl Zeiss LSM710 laser scanning microscope system at a magnification of ×400 as described previously [21]. 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 ×400 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 g) were used for CED as described previously [23]. A total volume of 5 µl (5.6 µg of siRNA or 0.62 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 RNA-later (Invitrogen, Paisley, UK).

2.13. qRT-PCR

Rat brains were collected in RNAlater (Invitrogen, Paisley, UK) and total RNA was obtained from them. Total RNA sample underwent DNase treatment (Invitrogen, Paisley, UK) to eliminate any degradation of RNA. Prior to reverse transcription, each sample was 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, and 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 MicroElute™ PCR Purification Kit (Qiagen) following the manufacturer’s instructions.

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 PreCellex Homogeniser (Stratton Scientific, Stratton, Derbshire, 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: 1:5,000) for 1 h at room temperature. Membranes were then rinsed in PBS-T thoroughly at room temperature before visualization 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).

3. Results

3.1. Development and biophysical characterization of anionic nanoparticles

LPR (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 PRLAP1 and DOPE-PEG2000 in PRLAP2. 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). PRLAP2 formulations were bigger than both PRLAP1 and non-PEGylated PRLA (Fig. 1A) while the charges of both PEGylated nanocomplexes were less anionic than non-PEGylated nanocomplexes (e.g. −25 mV for PRLAP1 vs −40 mV for PRLA at 4:3:1 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...
achieved in the absence of serum, whereas when serum was added the quenching was only reduced by 1.2-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 PRLA 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 PRLA at 95% quenching, packaged the siRNA better than PEGylated PRLAP1 and PRLAP2 that both quenched siRNA fluorescence by approximately 85% (Fig. 1D). In addition, PRLA was the least sensitive formulation to heparin as it achieved 50% dissociation at 0.21 U/ml heparin compared to PRLAP1 and PRLAP2 that both required 0.13 U/ml heparin. All formulations achieved 100% dissociation following heparin challenge at higher concentrations (0.5 U/ml for PRLA and 1.0 U/ml for both the PRLAP1 and PRLAP2).

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 PRLAP2 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 (PRLAP1 and PRLAP2) and non-PEGylated (PRLA) formulations at 4:3:1 molar charge ratios were compared for silencing of luciferase in transfections of Neuro-2A-Luc cells (Fig. 3A). PRLAP1 was significantly better by approximately 2.1-fold and 1.8-fold (p < 0.01 in both cases), than PRLAP2 and PRLA at 100 ns, respectively and was only inferior to L2K at 50 ns (p < 0.05). The same formulations were compared for silencing of GAPDH enzymatic activity in 1HAEo-cells (Fig. 3B). Again PRLAP1 was significantly better by more than 2-fold (p < 0.001 in both cases), than PRLAP2.
and PRLA at 100 nM, respectively, with no significant differences compared to L2K at 50 nM and 100 nM. In addition, when we compared PRLAP1 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) PRLAP1 resulted in 60% silencing, which was significantly better than either PRLAP2 (p < 0.05) or PRLA (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 PRLAP1 only at 10% serum (p < 0.01).

Anionic PRLA, PRLAP1 and PRLAP2 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, PRLA, and PRLAP2 but was even more pronounced with PRLAP1 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. PRLAP1 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.
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
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. 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, PRLA, PRLAP1 and PRLAP2 complexes with siRNA targeting GAPDH. UNTR = untreated cells. Scale bar = 20 μm.
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 nanocomplexes of ~110 nm were similar (PRLAP1) or slightly larger (PRL AP2) 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].

Fig. 5. In vivo silencing of BACE1 following CED administration of PEGylated nanoparticles into rat striatum. A) Anionic PEGylated PRLAP1 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 PRLAP1 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 PRLAP1 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 PRLAP1 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 2–5: BACE1 siRNA treated rats.
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 PRLAP1 and PRLAP2 resulted in more efficient packaging of siRNA than the non-PEGylated PRLA. We then tested the nanoparticle stability in turbidity assays using 50% mouse serum to better reflect in vivo conditions [32]. PEGylated and non-PEGylated PRLA 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. PRLAP1-mediated silencing of 60–80% that was significantly better than PRLAP2 and non-PEGylated PRLA for both luciferase and the endogenous GAPDH gene in both, serum-containing or serum-free conditions PRLAP1 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, PRLA 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 PRLAP1 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, PRLAP2 was slightly bigger than PRLAP1 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 PRLAP1 and PRLAP2 can only be explained by the fact that the PEGylated lipid moiety in PRLAP1 is a C16 saturated acyl chain while in PRLAP2 it is a C18 unsaturated acyl chain. The effectiveness of lipids with shorter acyl 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. PRLAP1 resulted in about a 60% reduction in BACE1 mRNA 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 PRLAP1-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 PRLAP1 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 material related to this article can be found at [http://dx.doi.org/10.1016/j.biomaterials.2014.06.003](http://dx.doi.org/10.1016/j.biomaterials.2014.06.003).

**References**


