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Multifunctional receptor-targeted nanocomplexes for the delivery of therapeutic nucleic acids to the Brain☆

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ABSTRACT

Convection enhanced delivery (CED) is a method of direct injection to the brain that can achieve widespread dispersal of therapeutics, including gene therapies, from a single dose. Non-viral, nanocomplexes are of interest as vectors for gene therapy in the brain, but it is essential that administration should achieve maximal dispersal to minimise the number of injections required. We hypothesised that anionic nanocomplexes administered by CED should disperse more widely in rat brains than cationics of similar size, which bind electrostatically to cell-surface anionic moieties such as proteoglycans, limiting their spread. Anionic, receptor-targeted nanocomplexes (RTN) containing a neurotensin-targeting peptide were prepared with plasmid DNA and compared with cationic RTNs for dispersal and transfection efficiency. Both RTNs were labelled with gadolinium for localisation in the brain by MRI and in brain sections by LA-ICP-MS, as well as with rhodamine fluorophore for detection by fluorescence microscopy. MRI distribution studies confirmed that the anionic RTNs dispersed more widely than cationic RTNs, particularly in the corpus callosum. Gene expression levels from anionic formulations were similar to those of cationic RTNs. Thus, anionic RTN formulations can achieve both widespread dispersal and effective gene expression in brains after administration of a single dose by CED.

1. Introduction

Genetic therapies involve the enhancement, replacement, modification, regulation and silencing of gene expression and offer great promise for the treatment of a wide range of diseases, of the central nervous system (CNS), including neurodegenerative, neuromuscular and metabolic diseases as well as cancers, many of which are currently untreatable [1–5]. Safe, but efficient delivery of therapeutic nucleic acids, however, remains a major technological barrier to the development of clinical therapeutics of the CNS. Nanocomplexes for gene delivery are of interest as alternatives to viral vectors as they can package a wider range of nucleic acids ranging from siRNA molecules of 20 or so nucleotides to tens of kilobases of plasmid DNA, and are less immunogenic than viruses allowing more effective repeated dosing of gene therapies [6,7]. Nanocomplexes may be delivered to the brain by the systemic route or by direct injection. Systemic delivery is limited in efficacy by the almost impermeable nature of the blood brain barrier (BBB) and rapid clearance of nanocomplexes from the circulation by the reticuloendothelial system (RES), particularly in the liver [8–10]. Direct injection methods such as intraparenchymal, intracerebroventricular and intrathecal injection, depend on diffusion for drug dispersal and so are limited in their dispersal by drug concentration and require injections at multiple sites to achieve widespread coverage of the brain.

In recent years, convection-enhanced delivery (CED) has been shown to achieve widespread distribution of therapeutics in the

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brain from a single administration [11], including viral gene therapy vectors [12–14]. CED utilises extremely fine intracranial catheters, implanted directly into the brain or spinal cord and distributes therapeutic agents along a pressure gradient generated between the catheter tip and the extracellular space, achieving controlled, homogeneous distribution of drugs over distances of up to 5 cm from the catheter tip in human brains [15]. Clinical trials involving administration of nanoparticles for gene therapy into the brain by CED have already been performed in patients with primary brain tumours [16,17], but more efficient formulations are required that achieve widespread dispersal and therapeutic delivery from a single administration.

Previous studies have shown that for widespread dispersal in the brain by CED, nanoparticles should be anionic or neutral rather than positively charged [18–20] and less than 200 nm [20]. Anionic liposomal complexes, however, have not been developed as extensively as cationic gene delivery complexes due to poor packaging of DNA and poor transfection efficiency [21,22]. In recent studies nucleic acid packaging into anionic complexes has been improved by various strategies, one of which involved combining anionic liposomes with polycationic protamine as an electrostatic bridge between the liposome and the nucleic acid [23,24]. In this study we have used a similar strategy to formulate an anionic receptor-targeted nanocomplex (RTN) comprising a mixture of a peptide containing a cationic oligolysine domain for DNA packaging and a neurotensin, receptor-targeting domain, and an anionic liposome. A similar cationic RTN formulation described previously [25] was also prepared containing the same peptide and plasmid, but a cationic liposome instead of an anionic liposome. In this study anionic and cationic RTNs, labelled with a gadolinium contrast agent and a rhodamine fluorophore, were compared for their biophysical properties then administered to rat brains by CED and their distribution analysed by MRI in whole brain and in tissue sections by LA-ICP-MS and fluorescence microscopy. Transgene expression was assessed by qRT-PCR and fluorescence microscopy for green fluorescent protein (GFP) reporter gene expression.

2. Materials and methods

2.1. Materials

Lipids (Supplementary Table 1); 1,2-dioleoyl-sn-glycero-3-phospho-(1′-rac-glycerol) (DOPG), 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP), 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine-N-(lissamine rhodamine B sulfonyl) (DOPE-Rhodamine) and 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE) were purchased from Avanti Polar Lipids Inc. (Alabaster, Alabama, USA). GdDOTA(GAC_{12})$_2$ was synthesised as described by Kielar et al. [26]. Neurotensin (Nt) targeting peptide, its scrambled version (NtS) and the control peptide K_{16} (Supplementary Table 2) were synthesized on a MultiSynTech Syro peptide synthesizer using commercially available Fmoc amino acids (Novabiochem, Nottingham, UK) and standard automated protocols, as described previously [25]. The plasmid pCI-Luc consists of the luciferase gene from pGL3 (Invitrogen, Paisley, UK) subcloned into pCI (Promega, Southampton, UK). The plasmid pEGFP-N1 (4.7 kb) containing the gene for enhanced green fluorescent protein (GFP) was obtained from Clontech (Basingstoke, UK). 100 nm polystyrene nanospheres were purchased from Phosphorex Inc. (Hopkinton, MA, USA) with both cationic (+48.1 mV, orange Ex/EM 520/540 nm) and anionic (−47.9 mV, blue Ex/Em 360/440 nm) charges. The oligonucleotide primers and standards for qRT-PCR were provided by qStandard (Middlesex, UK) and were as follows: eGFP: forward primer 5′-CTTCAAGATCCGC CACAACAT-3′ and reverse primer 5′-GGTGCTCAGGTAGTGGTTGTC-3′; Rpl13: forward primer 5′-CCCTACAGTTAGATACCACACCAA-3′ and reverse primer 5′-GATAC-CAGCCACCCTGAGC-3′; Beta actin: forward primer 5′- ACGGTCAGGTCATCACTATCG-3′ and reverse primer 5′-AGCCACCAATCCACACAGA-3′; Sdha: forward primer 5′-TGGACCTTGTCGTCTTTGG-3′ and reverse primer 5′-TTTGCCTTAATCGGAGGAAC-3′.

2.2. Liposome formulation

Liposomes were formulated with lipid mixtures at specific molar ratios as follows; cationic liposomes DOTAP:DOPE:DOPE-Rhodamine:GdDOTA(GAC_{12})$_2$ and anionic liposomes DOPG:DOPE:DOPE-Rhodamine:GdDOTA(GAC_{12})$_2$ both at a molar ratio of 35:49:1:15 mol% respectively. Liposomes were prepared by dissolving the individual lipids in chloroform at 10 mg/mL and mixing them together, followed by rotary evaporation to produce a thin lipid film. Lipids were then rehydrated with sterile water whilst rotating overnight and then sonicated for an hour in a water bath to reduce the size to unilamellar liposomes.

2.3. Nanocomplex formulation and biophysical characterisation

LPD nanocomplex formulations were prepared by mixing aqueous solutions of anionic liposome (L), peptide (P) and plasmid DNA (D) at charge ratios of 3:2:1 (14.1:1.15:1 weight ratio) for anionic formulations and 0.5:5:1 (2.35:2.9:1 weight ratio) for cationic formulations, diluted to 0.01 mg/mL (DNA) in OptiMEM (Invitrogen, Paisley, UK) for *in vitro* transfections, diluted to 0.005 mg/mL (DNA) in sterile water for biophysical characterisation and diluted to 0.32 mg/mL (DNA) in sterile water for *in vivo* experiments. Six nanocomplex formulations were produced (Table 1), with a targeting peptide neurotensin (Nt), a scrambled neurotensin (NtS) and a non-targeting K_{16} peptide. Size and charge of liposomes, nanocomplexes and nanospheres was analysed using a Malvern Nano ZS (Malvern, UK) at a temperature of 25 °C, viscosity of 0.89 cP and a refractive index of 1.33.

2.4. In vitro transfections

The murine neuroblastoma cell line Neuro-2A (ATCC, Manassas, VA, USA) was maintained in Dulbecco's Modified Eagle Medium, 1% non-essential amino acids, 1 mM sodium pyruvate and 10% FCS (Invitrogen, Paisley, UK) at 37 °C in a humidified atmosphere in 5% carbon dioxide. Cell transfections were performed as previously described [25], briefly, cells were seeded at 2×10^4 per well in 96-well plates in 175 μL of complete. 24 h later 25 μL of the nanocomplex formulations (Table 1) in OptiMEM, containing 0.25 μg of plasmid DNA was added to the cells in replicates of six. Plates were centrifuged at 1500 rpm for 5 min (400× g) and incubated for 24 h at 37 °C. Cells were then lysed and a chemiluminescence assay performed to measure transfected luciferase activity (Promega, Southampton, UK) and protein concentration determined using a Bio-Rad protein assay (Hemel Hempstead, UK). Luciferase activity was expressed as RLU per milligram of protein. Cell viability assays were performed with the CellTiter 96 Aqueous One Solution Cell Proliferation Assay (Promega, Southampton, UK). Luciferase, protein concentration and toxicity measurements were performed in an Optima Fluostar microplate reader (BMG Labtech, Aylesbury, UK).

2.5. In vivo Brain delivery

All animal experiments were carried out with licences issued in accordance with the United Kingdom Animals (Scientific Procedures) Act 1986 (UK). For all experiments male Wistar rats (B&K Universal, Hull, UK) were anaesthetised and placed in a stereotactic frame, burr holes were drilled to allow cannula implantation to corpus callosum on the left and striatum on the right hand side of the brain was via a 220 μm outer diameter fused silica cannula at a rate of 0.5 μL/min at each site (2.5 μL for corpus callosum and 5 μL for striatum) using an infusion pump (World Precision Instruments, Inc, Sarasota, FL, USA). Following infusion, the

Table 1
LPD nanocomplex composition and associated size and zeta potential. Measurements were taken immediately after formation and 150 days post, as measured by dynamic light scattering.

LPD Nanocomplex	Charge Ratio (L:P:D)			Size (nm)		Zeta PD (mV)	
	Liposome	Peptide	DNA	Day 0	Day 150	Day 0	Day 150
Cat K_{16}	Anionic Liposome (0.5)	K_{16} (5)	pCI-Luc/eGFP (1)	196.5 (±4.4)	147.8 (±2.6)	+36.1 (±0.5)	+37.4 (±0.6)
Ani K_{16}	Anionic Liposome (3)	K_{16} (2)	pCI-Luc/eGFP (1)	170.6 (±7.1)	159.5 (±3.0)	−44.4 (±4.2)	−61.1 (±0.5)
Cat NtS	Anionic Liposome (0.5)	NtS (5)	pCI-Luc (1)	181.1 (±4.5)	153.2 (±2.9)	+31.5 (±0.7)	+31.3 (±2.2)
Ani NtS	Anionic Liposome (3)	NtS (2)	pCI-Luc (1)	254.0 (±3.3)	182.8 (±5.2)	−61.8 (±2.2)	−55.5 (±0.8)
Cat Nt	Anionic Liposome (0.5)	Nt (5)	pCI-Luc/eGFP (1)	216.9 (±6.8)	172.4 (±2.1)	+30.0 (±1.1)	+31.6 (±0.5)
Ani Nt	Anionic Liposome (3)	Nt (2)	pCI-Luc/eGFP (1)	177.6 (±1.4)	150.1 (±5.8)	−61.6 (±6.5)	−65.4 (±3.3)

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