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Development and characterization of self-assembling nanoparticles using a bio-inspired amphipathic peptide for gene delivery

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ABSTRACT

The design of a non-viral gene delivery vehicle capable of delivering and releasing a functional nucleic acid cargo intracellularly remains a formidable challenge. For systemic gene therapy to be successful a delivery vehicle is required that protects the nucleic acid cargo from enzymatic degradation, extravasates from the vasculature, traverses the cell membrane, disrupts the endosomal vesicles and unloads the cargo at its destination site, namely the nucleus for the purposes of gene delivery. This manuscript reports the extensive investigation of a novel amphipathic peptide composed of repeating RALA units capable of overcoming the biological barriers to gene delivery both *in vitro* and *in vivo*. Our data demonstrates the spontaneous self-assembly of cationic DNA-loaded nanoparticles when the peptide is complexed with pDNA. Nanoparticles were <100 nm, were stable in the presence of serum and were fusogenic in nature, with increased peptide α-helicity at a lower pH. Nanoparticles proved to be non-cytotoxic, readily traversed the plasma membrane of both cancer and fibroblast cell lines and elicited reporter-gene expression following intravenous delivery *in vivo*. The results of this study indicate that RALA presents an exciting delivery platform for the systemic delivery of nucleic acid therapeutics.

1. Introduction

The development of an efficient non-viral gene delivery vehicle has become increasingly desirable to circumvent the limitations associated with viral vector mediated gene delivery, namely, toxicity, limited nucleic acid loading capacity, immunogenicity and high costs associated with large scale production of such vectors. The design of non-viral gene delivery vehicles has focused on the ability to condense nucleic acids into particles with optimal characteristics for cellular uptake, specifically, cationic particles within the nanometer range which can successfully protect the cargo from degradation and release it at the desired delivery site. Ease of manufacture with respect to scale up, storage and toxicity are also key considerations that must be addressed prior to any clinical application.

The use of synthetic peptides for gene delivery presents an attractive option. Solid-state syntheses together with HPLC techniques confer almost complete control over the composition of the peptide [1]. Viral genomes provide an ideal resource when it comes to the design of such bio-inspired peptides. For example, the 27 amino acid MPG peptide is derived from the fusion domain of HIV gp41, which enables cellular entry, and the nuclear import sequence of SV40 T antigen, which binds the cargo, also facilitating entry into the nucleus [2]. The formation of polyplexes with the MPG peptide is dependent on counter-ion condensation, and studies have shown that this peptide can deliver both siRNA and DNA *in vitro* after just 1 h [2,3]. Further studies have streamlined the MPG to the 21mer MPG-8 with successful knockdown of the Cyclin B1 gene following systemic administration [4].

Perhaps one of the most revolutionary peptides is GALA; this is fusogenic in nature and the seven EALA repeats confers α-helicity linked to pH responsiveness, realized through excellent endosomal disruption [5,6]. Due to the spacing of the charged residues (every fourth amino acid) this peptide is capable of taking on the conformation of an amphipathic α-helix at a lower pH [5]. However, due to its anionic nature, it cannot condense and protect nucleic acids [6–8]. Addition of the GALA peptide to cationic complexes however, has proven successful at facilitating the endosomal escape of multi-functional delivery systems which are capable of binding and delivering nucleic acids intracellularly [9–11].

To that end KALA was the first artificially designed peptide of its kind with the glutamate residues of GALA replaced with lysine residues, enabling nucleic acid condensation and protection [12]. The presence of multiple lysine residues in KALA means that in contrast to GALA, which always has a higher activity at lower pH, KALA's ability to permeabilize membranes is therefore dependent on both composition and pH

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[12,13]. The net charge of KALA increases with decreasing pH in the presence of neutral liposomes disrupting the α-helical structure and ablating any membrane permeabilizing activity. In the presence of negatively charged membranes however, the increased positive charge on KALA allows improved binding to the membrane and enhances its membrane permeabilizing activity [12]. However, KALA's α-helicity in a broad range of pH environments contributes to toxicity observed with the peptide. Endosomolytic agents should only become active in an acidic pH in order to prevent the disruption of other cellular membranes such as the plasma membrane and the membranes of intracellular organelles and it is this loss of specificity which can be cytotoxic [14].

KALA has however been integrated into a number of gene delivery systems to aid endosomolysis of the complexes. PEGylated KALA has been used to coat polyethylenimine/pDNA complexes to prevent the aggregation of nanoparticles and enhance endosomal disruption [15], as well as being incorporated into a tetra-lamellar multi-functional envelope-type nano device (T-MEND) yielding transfection efficacy's 20-fold higher in dendritic cell cultures (JAWS II) compared with the non-modified T-MEND [16].

Arginine is found in naturally occurring DNA binding/condensing motifs with a higher frequency than lysine and has been shown to be a superior transfection agent [17–19]. As arginine-rich peptide sequences exhibit improved internalization they have been widely exploited to aid the delivery of various gene delivery vehicles including peptide dendrimers [20], liposomes [21] and polymer systems [22–24].

In this study, we therefore designed and tested a modified peptide termed RALA, where the lysine residues were replaced with arginine, with the rationale that we could decrease cytotoxicity by retaining pH sensitivity, while potentially improving binding to the negatively charged outer leaflets of membranes and nucleic acids (Fig. 1). Through a series of biophysical, *in vitro* and *in vivo* studies, we demonstrate that RALA is indeed a highly effective DNA delivery platform with potential for ultimate translation of this delivery platform into the clinic.

2. Materials and methods

2.1. Materials

2.1.1. RALA peptide

The RALA peptide (Fig. 1) was produced by solid-state synthesis (FMOC) (Biomatik, USA) and supplied as a desalted lyophilized powder. This necessitated reconstitution before use. After cleavage from the resin the desired products were purified by and confirmed by RPHPLC. The molecular mass was confirmed as 3327.98.

2.1.2. Plasmids

pEGFP-N1 was purchased from Clontech (USA) and pCMV-Red Firefly Luc was purchased from Addgene (USA). Plasmids were propagated in MAX Efficiency® DH5α™ Competent Cells (Life Technologies, UK), purified using PureLink® HiPure Plasmid Filter Maxiprep Kit (Life Technologies, UK) and quantified by UV absorption at 260 nm.

2.1.3. Cell lines

ZR-75-1 human breast cancer, PC-3 human prostate cancer and NCTC-929 murine fibroblast cell lines were obtained from the American Type Culture Collection. All cell lines were authenticated by short tandem repeat (STR) profiling carried out by the suppliers and routine testing revealed that these cells were *Mycoplasma*-free.

The sequence of RALA
N-WEARLARALARALARHLARALARALRACEA-C

Fig. 1. The 30 amino acid sequence of RALA. The hydrophilic arginine (R) domain facilitates nucleic acid binding while the hydrophobic leucine (L) region interacts with lipid membranes. These two regions are separated by alanine (A) rich regions which give the peptide its amphipathicity. Tryptophan behaves as a spectroscopic probe while the glutamic acid residues at each terminus enhance solubility in water at a physiological pH.

2.1.4. Animals

Female, six-week-old C57BL/6 mice were purchased from Charles River Laboratories. The animals were housed in an open facility at 21 °C and 50% humidity with food and water *ad libitum*. The experimental protocols were compliant to the UK Scientific Act of 1986 and project license 2678.

2.2. Methods

2.2.1. Preparation of RALA/pDNA complexes

The complexes were prepared at N:P ratios (the molar ratio of positively charged nitrogen atoms in the peptide to negatively charged phosphates in the pDNA backbone) ranging from 1 to 12 by adding appropriate volumes of peptide solution to 1 μg pDNA. The complexes were incubated at room temperature for 30 min to allow the formation of the complexes. All complexes were used immediately following their preparation unless otherwise stated.

2.2.2. Gel retardation assay

Various quantities of the RALA peptide were added to 1 μg of pDNA to make complexes at N:P ratios 1–12. Samples were electrophoresed through a 1% agarose gel containing ethidium bromide with Tris-acetate running buffer at 80 V for 60 min and gel visualized using a Multispectrum Bioimaging System (UVP, UK). Image is the representative image of three independent studies.

2.2.3. Particle size and zeta potential analysis

RALA/pDNA complexes were prepared at N:P ratios 1, 2, 4, 6, 10, 12 and 14. These samples were diluted to 1 ml with distilled water before the measurement of particle size and zeta potential using a Nano ZS Zetasizer and DTS software (Malvern Instruments, UK).

2.2.4. Transmission electron microscopy (TEM)

RALA/pDNA complexes were prepared at a N:P ratio of 10 and a Formvar/Carbon mesh grid (Agar Scientific, UK) placed face down on a 10 μl sample of the complexes for 10 min. The loaded grid was dried overnight and stained for 5 min with 5% aqueous uranyl acetate at room temperature. The grid was imaged using a JEOL 100CXII transmission electron microscope at an accelerating voltage of 80 kV.

2.2.5. Encapsulation assay of pDNA within RALA complexes

RALA/pDNA complexes were prepared at N:P ratios 1, 2, 4, 6, 10, 12 and 14. 50 μl samples of each N:P ratio were pipetted into a 96-well plate. As per manufacturer's instructions stock Quant-iT™ PicoGreen® Reagent (Life Technologies, UK) was diluted 1:200 in TAE buffer and 50 μl was added to each sample. Sample fluorescence was analyzed by excitation at 480 nm and the fluorescence emission intensity measured at 520 nm using a Synergy 2 Multi-Mode Microplate Reader (BioTek Instruments Inc., UK).

2.2.6. Serum stability

RALA/pDNA complexes were prepared at a N:P ratio of 10. The complexes were incubated for 1, 2, 3, 4, 5 and 6 h at 37 °C in the presence and absence of 10% fetal calf serum. Subsequently, Sodium Dodecyl Sulfate (SDS) (Sigma-Aldrich, UK) was added (10%) to decomplex the pDNA from RALA. Following incubation for 10 min, samples were electrophoresed through a 1% agarose gel containing ethidium bromide with Tris-acetate running buffer at 80 V for 60 min and visualized using a Multispectrum Bioimaging System (UVP, UK). Image is the representative image of three independent studies.