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The interplay between cationic peptide sequence, siRNA and electrolyte: formulating lipopolyplexes in NaCl solution affords nanocomplexes with exceptional levels of gene silencing

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Lipopolyplexes are important non-viral vectors for the delivery of therapeutic siRNA. However, many formulations developed are unstable or show poor cellular uptake under physiologically relevant conditions, for example in the presence of serum. In this work, we have studied the effects of formulating ternary lipopolyplexes in NaCl solution on nanocomplex structure and gene silencing activity. The lipopolyplexes were prepared from vesicles containing a 1 : 1 mixture of the cationic lipid DOTMA and the helper lipid DOPE, co-formulated with singly branched cationic peptides attached to a targeting sequence and with siRNA, in sodium chloride solution. These lipopolyplexes retained high levels of gene silencing in the presence of media supplemented with fetal bovine serum. Exceptionally good activity was observed when His- or His-Arg cationic sequences were used, superior to control formulations with Lipofectamine 2000. We have shown that these lipopolyplexes are large, multilamellar complexes which efficiently encapsulate and protect siRNA. In addition, the most effective His- or His-Arg cationic sequences showed looser complexation between the cationic peptide and the siRNA. This work thus represents a significant advance in the optimisation of lipopolyplex formulations for therapeutic gene silencing *in vivo* under physiologically relevant conditions, paving the way for more effective and less toxic siRNA-based therapies for clinical use.

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Introduction

The last five years has highlighted the crucial role of RNA-based therapies in preventing or treating life-threatening diseases. Key to the success of these new therapies has been the development of nanoparticle formulations that can encapsulate, protect and deliver messenger RNA (mRNA) and small interfering RNA (siRNA). The most striking examples of these are the lipid nanoparticle (LNP) formulations that enabled the development of highly effective mRNA-LNP vaccines against SARS-CoV-2, developed by BioNTech/Pfizer and Moderna in response to the global threat of COVID.[1,2] Significant advances in the delivery of siRNA-based therapeutics for gene knockdown and silencing have also been made recently.[3,4] The first siRNA-LNP drug, Onpattro, was approved by the FDA in 2018 for the treatment of a fatal familial genetic defect, transthyretin (TTR) mediated amyloidosis, and several other siRNA-LNP formulations are currently in clinical trials for the treatment of various cancers.[1,4] Lipopolyplex (LPR) formulations have also attracted significant interest as non-viral vectors for delivery of siRNA.[5–7] These are ternary complexes of lipids (L), cationic polymers or cationic peptides (P) and siRNA (R). Their flexible and modular formulation has some advantages over LNP complexes. They have greater thermal stability, are often smaller in size and have the potential to display cell-specific targeting ligands at their surface.

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Effective RNA silencing[8] by lipid-based nanoparticles requires siRNA to be selectively delivered to target cells; efficiently internalised, usually by endocytosis; and subsequently released from the endosome into the cytoplasm to interact with the multi-protein RNA-inducing silencing complex (RISC).[9–11] LPR formulations containing cationic polymers such as poly(ethyleneimine) (PEI) or poly(amido amine) (PAMAM) are very effective at complexing siRNA, are taken into cells efficiently and can escape from endosomes *via* the proton sponge effect. However, their strong affinity for siRNA means that the cargo is subsequently not released rapidly into the cytoplasm;[12] moreover, these cationic polymers have undesirable toxicity, limiting their clinical use.[13] Cationic peptides can also similarly bind siRNA through electrostatic interactions. They have the advantages of being biodegradable, biocompatible and easy to synthesise; in addition, they have low toxicity. Furthermore, the peptide sequences used can be varied to confer a range of functionalities, such as pH responsiveness or receptor targeting, on the resulting LPRs. For example, LPRs have been formulated with slightly acidic pH-sensitive peptides (SAPSP) which are primed to release the siRNA cargo at cytoplasmic pH.[14] Multifunctional peptides, incorporating cationic ($(Arg)_9$), fusogenic and gastrin-releasing peptide receptor targeting sequences, have been formulated into LPRs to selectively deliver siRNA to prostate cell lines.[15] LPRs incorporating pH responsive peptides have also been used to deliver siRNA to tumours, aided by focused ultrasound.[16] Finally, LPR nanoparticles with four-branched His-Lys cationic peptides have also been used to deliver mRNA to MDA-MB-231 tumour cells.[17] However, there is still a real need to develop nanocomplexes of both siRNA and mRNA which are stable both in the presence of serum and at a variety of in-use temperatures, and which can be effectively targeted to specific cell types.

We have previously investigated ternary lipid–peptide-pDNA lipopolyplexes (LPD) as gene delivery vectors.[18] The lipid components comprised mixtures of cationic lipids, particularly trimethyl [2,3-dioleyloxy-propyl] ammonium chloride (DOTMA) and analogues, with the helper lipid dioleoylphosphatidylethanolamine (DOPE) to promote endosomal release. The trifunctional peptides included sequences targeting receptors at the surface of human airway epithelial cells[19] or tumour cells;[20,21] a linking sequence (RVRR) which can be cleaved by endosomal furin, promoting nanoparticle disassembly;[22,23] and cationic sequences of Lys, His and Arg residues to condense the pDNA.[24,25] We have also explored the effects of different modalities of bioconjugation of targeting peptides on LPD transfection efficiency and structure.[26] Importantly, we have also shown that formulation of LPDs in the presence of NaCl solution results in nanoparticles that are stable in serum.[25] These retained high levels of transfection activity in the presence of physiologically relevant media, paving the way for these to be used in a clinical setting.

The design and formulation principles gained from these LPD studies can also be applied to LPR nanocomplexes. We have previously investigated lipopolyplexes formulated with siRNA, mixtures of DOTMA/DOPE and cationic peptides, as vectors for delivering siRNA to an alveolar cell line.[27] The cationic peptides included Lys, His, L-Arg, D-Arg and mixed His/Lys sequences, attached to a cell surface receptor sequence *via* the same furin-cleavable linker. We observed that the highest levels of knockdown were achieved with branched peptides containing mixed His/Lys sequences such as $(Lys\text{-}His\text{-}His\text{-}His)_4$. These are similar to the branched sequences investigated by Mixson and co-workers for the delivery of peptide-siRNA polyplexes[28] and peptide-mRNA-dioleoyl-3-trimethylammoniumpropane (DOTAP) lipopolyplexes.[17] Biophysical and structural studies showed that these peptide sequences resulted in nanocomplexes with looser binding to the siRNA, and a different geometry of siRNA : peptide interactions than with the less effective cationic sequences. We were also able to show, using confocal microscopy, that the siRNA was efficiently released into the cytoplasm from the mixed His/Lys sequences and persisted in the target cells. Importantly, although poor knockdown efficiencies were reported for LPRs formulated in water and then diluted with media, when we prepared these LPRs in OptiMEM, high levels of knockdown were observed. Whilst OptiMEM is not suitable for LPRs that will be used for clinical studies, these observations suggested that formulating LPRs in high salt conditions could be beneficial for both stability and knockdown efficiency.

Following from this work, and in the light of our successful preparation of stable targeted LPDs formulated from mixed His/Arg peptide sequences in high salt conditions, which show excellent transfection in serum,[25] here we report on the preparation, structure and knockdown capabilities of LPR nanocomplexes formulated from the same mixed His/Arg peptide sequences, and other cationic peptide sequences, in NaCl solution.

Materials and methods

Materials

DOTMA and DOPE were purchased from Tokyo Chemical Industry (Tokyo, Japan) and Avanti Polar Lipids (Alabama, USA) respectively. A549 cells (adenocarcinomic human alveolar basal epithelial) were obtained from ATCC (Manassas, USA). Human alveolar A549-Luc cells, stably transfected with an episomal S/MAR luciferase vector as described by Argyros *et al.*[29] were kindly donated by Professor Maya Thanou (Institute of Pharmaceutical Science, King's College London, UK). Custom-made siRNA (CUUACGCUGAGUACUUCGdTdT), denoted as Sigma siRNA, was from Sigma-Aldrich (Poole, UK) and was used for the preparation of samples requiring high amounts of siRNA, namely small angle neutron scattering (SANS), dynamic light scattering and zeta potential measurements. Silencers™ firefly luciferase (GL2 + GL3) siRNA (+siRNA) and Silences™ negative control siRNA (−siRNA) #1 were purchased from Ambion (USA). Invitrogen Lipofectamine and Lipofectamine 2000, Gibco 0.4% w/v Trypan blue in phosphate buffered saline (PBS), Gibco reduced serum medium OptiMEM™ and

PicoGreen™ reagent were supplied by Invitrogen Life Technologies (Paisley, UK). Luciferase assay kit was obtained from Promega (Southampton, UK). Bicinchoninic acid (BCA) protein assay kit was purchased from Fisher Scientific (Loughborough, UK). RPMI-1640 cell culture medium, 0.25% w/v trypsin/EDTA solution, fetal bovine serum (FBS), 1% v/v of 100x strength non-essential amino acids, 1% v/v of penicillin/streptomycin antibiotic solution (10 000 U mL^{-1}/10 mg mL^{-1}), L-glutamine were purchased from Sigma-Aldrich (Poole, UK), as were Ribonuclease A (RNAse A) from bovine pancreas, ribonuclease Inhibitor from human placenta known to specifically inhibit ribonucleases (RNAses) A, B and C (1), poly-L-aspartic acid sodium salt (pAsp), Trizma base, agarose, ethylenediaminetetraacetic acid disodium salt dehydrate (EDTA), boric acid, glacial acetic acid, bromophenol blue sodium salt, sucrose, magnesium chloride were obtained from Sigma-Aldrich (Poole, UK). GelRed nucleic acid gel stain (10 000× in water) was purchased from Biotium (Heyward, USA). Glacial acetic acid was purchased from VWR International Ltd (West Sussex, UK). EDTA (0.5 M, pH 8.0) solution was purchased from Promega (Southampton, UK). Tris-acetic-EDTA (TAE, pH 7.4) buffer and tris-borate-EDTA (TBE, pH 8.3) buffer were prepared from the above ingredients.

Peptide synthesis and purification

We have previously reported the synthesis and purification of the branched cationic peptides.[25] **H_{12}BLY** and **$(HR)_6$BLY** were subsequently re-synthesised by Biosynth Laboratories Ltd to enable additional SANS measurements.

Preparation of the lipopolyplexes

Vesicles composed of cationic DOTMA and the neutral helper lipid DOPE (at a 1 : 1 molar ratio) were prepared using a modification of the thin film method. Chloroform was removed from a 0.67 mg mL^{-1} solution of DOTMA and DOPE under vacuum overnight to leave a thin lipid film which was subsequently hydrated, with agitation, with ultrapure water (resistivity >18 Ω cm Thermo Scientific Barnstead water purification system EASYpure UV/UF, Loughborough, UK) with (Vs) or without (Vw) NaCl (up to 150 mM). In this way an initial vesicle suspension, with a concentration with respect to DOTMA of 1 mg mL^{-1}, was prepared. A more homogenous vesicle suspension was prepared from the initial vesicle suspension using a probe sonicator (SKL-950WT ultrasonic cell crusher, Ningbo Haishu Sklon Electronic Instrument Co. Ltd, Ningbo, China) fitted with a microtip operating at 30% of maximum output for 10 min at room temperature. When the vesicles were dispersed in a NaCl solution, the initial vesicle suspension was bath, rather than probe, sonicated (Fisherbrand precision general-purpose water bath, Loughborough, UK) operating at 80% of maximum output and 40 °C for 10 min. Vesicles prepared in water, OptiMEM™ and NaCl solution are denoted as Vw, Vo and Vs respectively.

To prepare LPRs, peptide solutions were first added to an equal volume of a sonicated suspension of vesicles composed of a 1 : 1 molar ratio of DOTMA : DOPE. Next an equal volume of siRNA solution was added, and the resulting mixture gently mixed to prepare the LPR. In all cases, a minimum of 15 min standing time of the LPR mixture at room temperature was allowed for complexation to occur before use. Unless otherwise stated, the LPRs were made at a lipid : peptide : siRNA charge ratio of 0.5 : 12 : 1. LPRs prepared entirely in NaCl solution are denoted as VsLPRs, whereas vesicles prepared in water while the LPR were formulated in OptiMEM™ are denoted as VwLPRo. All vesicles and LPRs were prepared freshly.

Cell culture

Luciferase-transduced A549 cells were maintained in RPMI-1640 media supplemented with 10% v/v of FBS, 1% v/v of 100× strength non-essential amino acids (NEAA), 1% v/v of 200 mM L-glutamine solution, and 1% of v/v penicillin/streptomycin solution (10 000 IU mL^{-1}/10 mg mL^{-1}) and kept at 37 °C in an incubator (Nuaire Autoflow CO_2 Air-Jacketed Incubator, Plymouth, USA) with 90% humidified atmosphere with 5% CO_2. The cells were fed on alternate days and passaged twice weekly when approximately 70% confluent using 0.25% trypsin-EDTA solution.

LPR knockdown

Immediately prior to knockdown with LPRs, luciferase-transduced A549 cells were seeded in 96-well transparent plates at a density of 1.2×10^4 cells per well for 24 h. On the day of the experiment, the medium was removed and 50 μL of either OptiMEM™ or RPMI-1640 media containing 10% v/v FBS were added to each well followed by the addition of 50 μL of LPR suspension at a siRNA concentration of 50 nM per well (25 μL of LPR diluted with 25 μL of OptiMEM or RPMI-1640 media containing 10% v/v FBS). LPRs were tested in triplicate. The cells were maintained at 37 °C in a 90% humidified and 5% CO_2 atmosphere for 24 h.

After incubation with the LPRs, the cells were rinsed with 50 μL of PBS (pH 7.0) and lysed with 50 μL of lysis buffer (200 mM Tris-HCl (pH 7.8), 2 mM EDTA and 0.05% v/v Triton X-100) for 1 h at room temperature. After this time, the cells were first frozen at −80 °C for 30 min and then defrosted and shaken in an orbital incubator for 1 h at room temperature. The luciferase activity was measured by transferring aliquots of cell lysate to a 96-well white plate and the luminescence measured using a FLUOstar Omega luminometer (BMG LABTECH GmbH, Ortenberg, Germany) using luciferase assay reagent (Promega Luciferase Assay Kit) in accordance with the manufacturer's protocol. Protein content was measured by adding a further aliquot of cell lysate to a 96-well transparent plate and mixed with protein assay reagent (Fisher Scientific BCA Protein Assay Kit) as per the manufacturer's instructions. The mixture was incubated at 37 °C for 30 min and the absorbance measured at 562 nm using a SpectraMax 190 plate reader (Molecular Device, USA). All measurements were performed in triplicate and the mean and SD calculated. The knockdown due to the LPRs was assessed by measuring the remaining luciferase activity, determined as relative light units (RLU) per milligram of protein (RLU per mg protein), in com-

parison to a negative siRNA control. A negative siRNA control was used for each group of samples. Lipofectamine 2000-siRNA complexes were used as positive control. The knockdown experiments were repeated on at least two, but frequently three separate occasions. In all cases the same trends in the data were seen, although the absolute level of transfection may have varied slightly between experiments.

Agarose gel retardation assay

To establish the complexation, release and protection afforded to siRNA by its entrapment in the LPRs, LPRs prepared in either water or a NaCl solution (final NaCl concentration of 120 mM) underwent agarose gel electrophoresis. To investigate complexation, 10 μL of LPR containing 0.10 μg of siRNA was mixed with 2 μL of gel loading buffer (containing 40% w/v of sucrose and 0.25% w/v of bromophenol blue). To study siRNA release, 10 μL of an LPR suspension containing 0.10 μg of siRNA was mixed with 0.625 μL of a 10 mg mL^{-1} aqueous solution of poly aspartic acid (pAsp) or 1.25 μL of a 1 mg mL^{-1} aqueous pAsp solution and mixed with 2 μL of the gel loading buffer. The protection afforded the siRNA by the LPR was determined by incubating 10 μL of LPR with 0.4 μL of a 0.1 mg mL^{-1} aqueous solution of RNAse A at 37 °C for 30 min, followed by 0.4 μL of an aqueous solution of RNAse inhibitor from human placenta (45 units per μL) at room temperature for 10 min to ensure complete deactivation of the RNAse A enzyme. 1.25 μL of a 1 mg mL^{-1} aqueous solution of pAsp was then added to the mixture to release any siRNA remaining associated with the LPRs for detection on the agarose gel, before mixing with 3 μL of gel loading buffer. After mixing with the gel loading buffer, each of the samples was loaded into wells in a 2.0% w/v of agarose gel in tris-borate-EDTA (TBE, pH 8.3) buffer containing 89 mM of Tris, 89 mM of boric acid, 2 mM of EDTA and 3 μL GelRed at 80 mV for 40 min (Fisher Brand, Model HU12 electrophoresis chamber, Loughborough, UK) and the resulting gel visualized under UV light illumination using an Alphalmager EP MultiImage Light Cabinet (Randpark Ridge, South Africa). Uncomplexed free siRNA and the enzyme-treated uncomplexed free siRNA were used as controls. Each gel was repeated on more than one occasion to ensure reproducibility.

PicoGreen fluorescence assay

A PicoGreen™ fluorescence assay was performed to establish the extent of siRNA complexation when contained in the LPR. A 50 μL aliquot of PicoGreen reagent (1 : 150 v:v PicoGreen in 3× Tris-EDTA buffer) was added to 100 μL of pre-formed LPR suspension prepared in either water or 154 mM NaCl, in black 96-well plates, and containing 0.2 μg of siRNA per well, and incubated at 5 min at room temperature. Note that, while the lipid : siRNA charge ratio was fixed at 0.5 : 1 for the experiment, the peptide : siRNA charge ratio varied in the range of 18–1 : 1 (*i.e.* 1 : 1, 2 : 1, 4 : 1, 6 : 1, 9 : 1, 12 : 1, 18 : 1). The fluorescence associated with the complexes in each well was measured using an excitation wavelength of 485 nm and an emission wavelength of 520 nm with a gain of 1000 using a FLUOstar Omega fluorimeter (BMG LABTECH GmbH, Ortenberg, Germany). Any unincorporated siRNA was quantified and expressed as relative fluorescence units (RFU) compared to the free (naked) siRNA control, which was denoted as 100% RFU to enable normalisation of the fluorescence signal. All experiments were performed in triplicate and the mean and SD value calculated.

Particle size and zeta-potential measurement of the LPR complexes

The apparent hydrodynamic size and zeta potential of the LPR in suspension (50 μL of LPR suspension containing 1.25 μg of Sigma siRNA) were measured using dynamic light scattering at a scattering angle of 173° (Malvern Zetasizer Nano ZS, Worcestershire, UK) at 25 ± 0.1 °C without dilution using a low-volume quartz cuvette, while the determination of ζ-potential was performed by diluting 50 μL of the LPR with either 900 μL of ultrapure double distilled water or a 150 mM NaCl aqueous solution and measuring using disposable capillary cells. The concentration of particles was sufficiently low to ensure the absence of any inter-particulate interactions. All samples were measured in triplicate and the mean and SD value of the apparent hydrodynamic size and the ζ-potential calculated.

Small angle neutron scattering of the LPR complexes

Small angle neutron scattering (SANS) experiments were used to establish the internal structure of freshly prepared LRs, LPRs and the vesicles from which they were made when prepared in water (and appropriate ratios of $H_2O : D_2O$) or in NaCl solution. The SANS experiments were performed using the instruments LoQ and SANS2D at the ISIS pulsed neutron source (Rutherford-Appleton Laboratories, Didcot, UK). All samples were measured in disk-shaped, fused silica banjo cells of 2 mm path length and thermostated at 25 °C. The scattering intensity, $I(Q)$, of the various samples was determined as a function of the scattering vector, $Q = (4\pi/\lambda)\sin(\theta/2)$ where $\sin(\theta/2)$ is the scattering angle, by normalizing the relevant sample scattering to the sample's transmission after subtraction of the scattering for the relevant solvent (with or without NaCl).

When fitting the data, a background correction was included to account for any mismatch in the level of incoherent and inelastic scattering between the sample and the deuterated solvent. The levels of the fitted backgrounds were routinely inspected to ensure that they were of a physically reasonable magnitude. Model-fitting of the SANS data for the systems analysed was performed using a (bespoke) *para*-crystalline lamellar stack model coded by Heenan (personal communication) for use with the SasView package.[30] This model accounts for scattering from a *para*-crystalline lamellar stack taking into account thirteen adjustable parameters, namely a flat background contribution to account for any inaccuracies in the solvent background subtraction, the *d*-spacing of the layers within the lamellar stacks, the layer thickness, the polydispersities of the *d*-spacing and layer thickness, a Lorentz

term (to model local deviations in bilayer surface curvature/rigidity and which was fixed at a constant value in the present study), the scale factors for unstacked layers ($N1$) and lamellar stacks containing up to five layers ($N2$–$N5$, where N is the number of bilayers/stack), and the scattering length densities of the bilayers and solvent.

The weighted fit between the measured and calculated scattering ($I(Q)_m$ and $I(Q)_c$, respectively) was achieved through application of the Levenberg–Marquardt algorithm,[31,32] with the various model parameters adjusted so as to reduce the calculated χ^2 value, obtained as:

$$\chi^2 = \frac{\sum_{i=1}^{n} \{I(Q)_m - I(Q)_c\}^2}{w_i^2}$$

where n is the total number of measured data points and w_i is the uncertainty on each $I(Q)_m$.

Results

Design of branched peptides

We have previously shown that lipopolyplexes formulated from singly branched cationic peptides were effective as both knockdown and transfection reagents, and that further branching of the cationic sequences reduced the activity.[24,25] We have also observed that longer cationic sequences give better results than shorter ones, and in this work, therefore, selected peptides containing a total of 24 His, Lys or Arg residues in the siRNA-binding region. All peptides contained a linker sequence (RVRR) designed to be cleaved by endosomal furin after internalisation of the nucleic acid complex,[22] together with a cyclic targeting sequence Y (CYGLPHKFCG) which was previously identified by phage display to bind human airway epithelial cells and to mediate gene delivery.[19]

There has recently been much interest in Arg-based[33] cationic peptides, lipopeptides and dendrimers for siRNA delivery, as Arg-containing sequences have cell-penetrating peptide properties and can also participate in bidentate hydrogen bonds with the phosphate backbone of siRNA.[34] There has also been much interest in His-based cationic peptides.[35] The isoelectric point of His residues (around pH 7.6) means that such peptides can act as "proton sponges" and promote escape from the acidic environment of the endosome: subsequently, in the neutral cytoplasm, these peptides exist as zwitterions and their binding to siRNA is reduced. However, there are remarkably few studies of mixed Arg-His or Arg-Lys cationic peptides being used for siRNA delivery.[36–40] As we had previously seen very good knockdown with LPR formulated from peptides containing mixed Lys-His sequences,[27] we therefore wanted to investigate the use of the mixed Arg-His cationic sequences in **(HHR)$_4$BLY** and **(HR)$_6$BLY**, and to compare these with LPR formulated from the mixed Lys-His sequence **(HK)$_6$BLY** and the mixed Arg-Lys sequence **(RK)$_6$BLY** (Fig. 1: Group 2 peptides). For comparison, we have also investigated LPRs formulated from branched cationic peptides of the same length, containing homo-Lys or homo-Arg or homo-His residues; the shorter **K$_4$BLY** peptide was included for comparison with previous studies (Fig. 1: Group 1 peptides).

In vitro knockdown experiments

Preliminary studies were carried out to determine the optimum formulation and incubation protocols for the luciferase knockdown experiments. Initially the shorter **K$_4$BLY** peptide was used to formulate VwLPRo nanocomplexes in

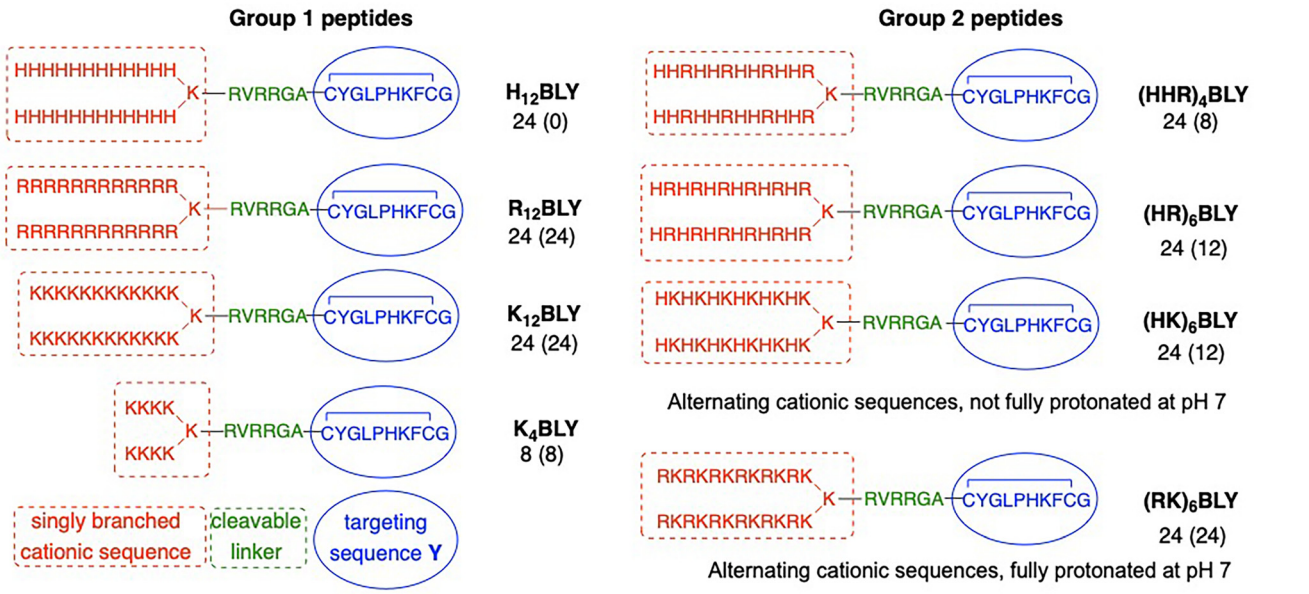


Fig. 1 Sequences of the singly branched cationic peptides used in this study. The number of cationic residues (and the number of those which are fully protonated at pH 7) in each sequence are indicated.

OptiMEM™. The gene silencing of luciferase in A549 epithelial cells expressing luciferase reporter using this VwLPRo formulation was compared with the knockdown efficiency afforded by a Lipofectamine 2000-siRNA complex and with naked siRNA. The optimum conditions (namely a balance between silencing activity and cell toxicity) were found to be with the LPR at an siRNA concentration of 50 nM per well, using the same order of mixing protocol as previously developed[27] and with a 24 h + 24 h incubation time (SI Fig. S1). Significantly, however, the knockdown achieved by LPRs was less sensitive to the order of mixing than the corresponding LPDs (SI Fig. S2), an observation which is most likely to be the consequence of the smaller molecular weight/increased diffusion coefficient of the siRNA compared to DNA and the fact that siRNA is not condensed before complexing. Subsequently, VwLPRw LPR formulations were prepared from all Group 1 and Group 2 peptides, and then diluted in OptiMEM™ and their ability to knockdown luciferase determined. From these knockdown studies, the optimum L : P : R charge ratio was found to be 0.5 : 12 : 1 (SI Fig. S3a). Encouragingly minimal toxicity was seen for cells treated with the LPR formulations (SI Fig. S3b and c).

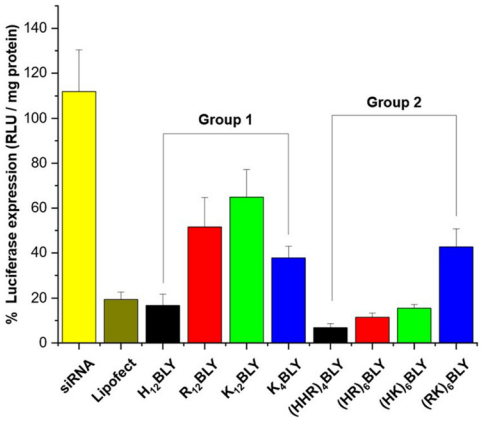
We next investigated the effects of using different aqueous media to prepare both the vesicles and the LPR nanocomplexes on knockdown efficiency. For LPR formulations to be developed for clinical use, they must be stable under physiological conditions and unfortunately many lipoplexes or polyplexes have significantly reduced activity in the presence of serum.[41,42] We have used RPMI-1640 media containing 10% v/v of fetal bovine serum (FBS) to mimic the biological environment. In addition, to prepare LPRs suitable for parenteral administration, they must be in a medium that is isotonic with body fluids, or else they may cause lysis of cells and pain upon administration. OptiMEM is isotonic with body fluids, and our preliminary studies suggested that formulation of LPR in OptiMEM gives nanocomplexes with high knockdown efficiencies in the presence of serum.[27] OptiMEM is designed as a reduced-serum medium and is not intended for use as a vehicle for injection. However, as OptiMEM contains electrolyte, we postulated that its presence was beneficial to nanocomplex stability and siRNA protection. To probe and compare the effect of formulation electrolyte on knockdown, we prepared a series of LPR formulations, as follows: vesicles prepared in water/LPR formulated in water (VwLPRw); vesicles prepared in water/LPR formulated in OptiMEM (VwLPRo); vesicles prepared in NaCl solution (120 mM NaCl)/LPR formulated in NaCl solution (VsLPRs). These were first diluted 1 : 4 with OptiMEM prior to incubation with the A549 cells (Fig. 2a). Reasonable levels of knockdown were observed with most of the Group 1 and Group 2 peptides for all three formulations. Particularly good results were seen with His-containing peptides, especially **H_{12}BLY**, **$(HHR)_4$BLY** and **$(HR)_6$BLY**; conversely the Arg- and Lys-containing peptides **R_{12}BLY**, **K_{12}BLY** and **$(RK)_6$BLY** were ineffective in most formulations. When these formulations were diluted 1 : 4 with RPMI-1640 media containing 10% FBS prior to incubation with the A549 cells (Fig. 2) the differences between the formulation methods became more apparent. Little or no knockdown was seen for any of the VwLPRw nanocomplexes, while only moderate levels were recorded for the VwLPRo. However, when the LPR were prepared at a final concentration of 120 mM NaCl solution, the VsLPRs formulated with **H_{12}BLY** and **$(HR)_6$BLY** gave knockdown levels of >80%. In order to determine why these combinations of cationic peptide sequence and formulation electrolyte give such striking activity in physiologically relevant media, we carried out a series of enzymatic and biophysical investigations.

RNAse A, which is present in FBS is known to digest siRNA reducing its gene silencing activity.[43] Consequently a gel electrophoresis study was performed to determine whether digestion of siRNA by RNAse A was a factor in the reduced knockdown observed when LPR were diluted in media (Fig. S4). Reassuringly, however, the observed reduction in knockdown in the luciferase-transduced A549 cells after incubation with VwLPRw/OptiMEM was not found to be attributed to degradation of siRNA by RNAse in the RPMI-1640 media containing 10% v/v FBS.

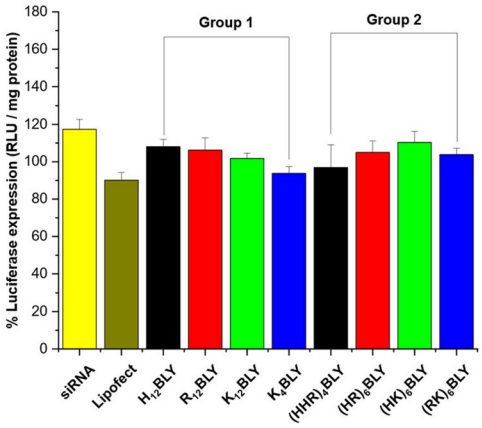
The ability of LPRs prepared fully in water (VwLPRw) and containing the various peptides to variously complex, to release and to protect siRNA was investigated using gel electrophoresis (Fig. 3). Lane A shows the complexing ability of the LPRs for siRNA, while the release and protection of siRNA within the LPRs are shown in Lanes B and C, respectively. For the VwLPRw, with the exception of faint bands present in the LPRs prepared using the **H_{12}BLY** and **$(HHR)_4$BLY** peptides, there was no band attributable to intact siRNA present in Lane A. This observation suggests that, with the exception of **H_{12}BLY** and **$(HHR)_4$BLY**, siRNA was completely complexed within the VwLPRw when using either Group 1 and Group 2 peptides. In Lane B, strong bands attributable to siRNA were present, indicating that siRNA was completely released from various LPRs upon the addition of pAsp. When naked siRNA was treated with varying concentrations of RNAse (Lanes C, C_1 and C_2), no band attributable to siRNA could be observed at any enzyme concentration. In contrast, however, when the LPRs were treated with the same concentration of RNAse followed by the addition of pAsp to release any siRNA, clear bands attributable to siRNA were observed. This observation suggests that siRNA was protected when complexed within the various VwLPRw.

LPRs prepared fully in an aqueous 120 mM NaCl solution (VsLPRs) were also examined using gel electrophoresis (Fig. 3). As with the VwLPRw, the VsLPRs exhibited the ability to both fully complex (Lane A) and release siRNA (Lane B). A faint band was observed for intact siRNA in Lane A for the LPR prepared using the **H_{12}BLY** peptide, but not for the LPR prepared with the **$(HHR)_4$BLY** peptide. Again, when the VsLPRs were treated first with RNAse and then with pAsp, a clear band corresponding to the siRNA was observed, suggesting that the siRNA is protected within the VsLPRs regardless of the peptide used for their preparation.

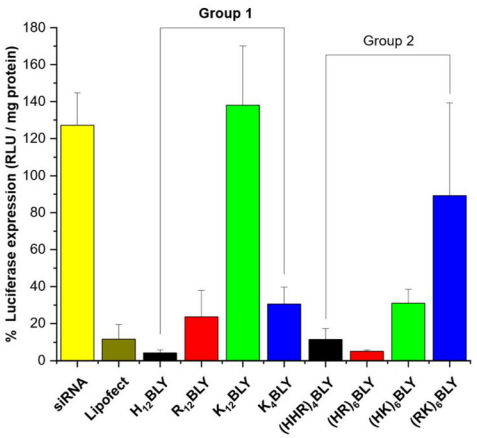
When naked siRNA was treated with the standard amount of RNAse A (*i.e.* 45 units μL^{-1}) a clear band attributable to



(a)(i) VwLPRw/OptiMEM



(b)(i) VwLPRw/Media



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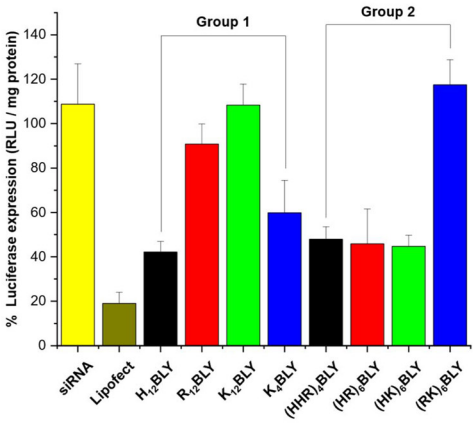

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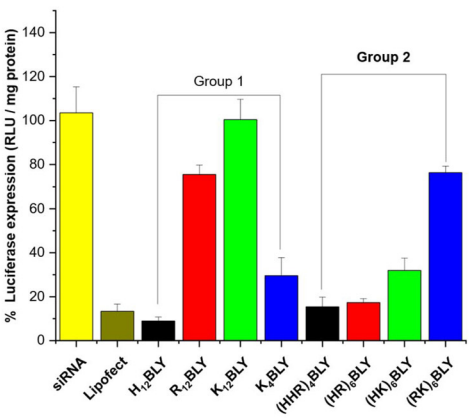
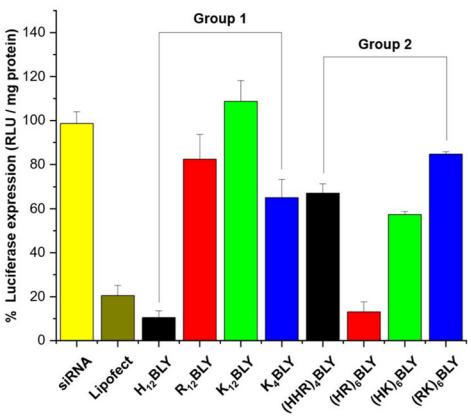


(a)(i) VsLPRs/OptiMEM



(b)(i) VsLPRs/Media

Fig. 2 Luciferase gene silencing activity achieved at 37 °C in luciferase-transduced A549 cells incubated with lipopolyplexes (LPRs) at a lipid : peptide : siRNA ratio of 0.5 : 12 : 1 incubated for 24 h + 24 h with luciferase-transduced A549 cells in the presence of (a) OptiMEM (LPRs diluted 1 in 4) or (b) RPMI-1640 media containing 10% v/v of FBS termed Media (LPRs diluted 1 in 4). Vw and Vs indicate that the lipid vesicles used to make the LPRs were prepared in water and a 120 mM NaCl solution, respectively while (i) VwLPRw, (ii) VwLPRo and (iii) VsLPRs mean that the LPRs were prepared in water, OptiMEM and a 120 mM NaCl solution, respectively. Cationic vesicles used to prepare the LPDs were composed of DOTMA : DOPE at 1 : 1 molar ratio. Note, the % difference in gene silencing activity for each experimental condition was obtained using negative control siRNA. Data are mean values ± standard deviation of three replicates.

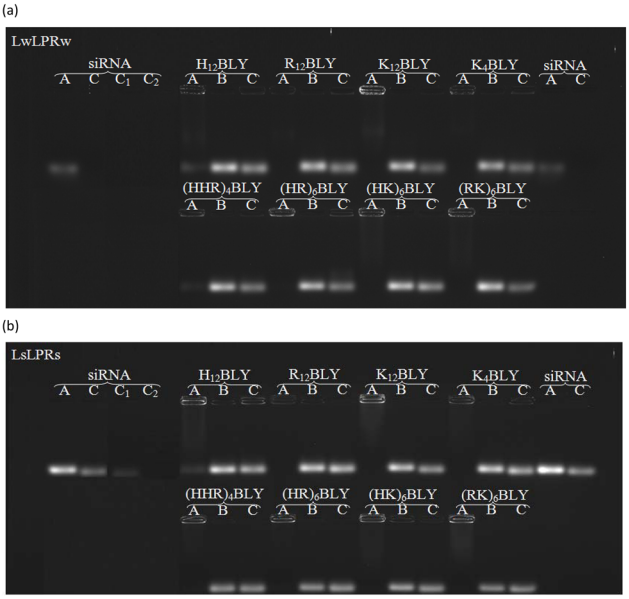


Fig. 3 Complexation, release and protection of lipopolyplexes (LPRs) using 0.01 mg mL^{-1} (–)siRNA. LPRs prepared at a L : P : R charge ratio of 0.5 : 12 : 1. Cationic vesicles composed of DOTMA : DOPE at a 1 : 1 molar ratio. Upper panel (a) shows VwLPRw, and the lower panel (b) VsLPRs. Complexation – Lane A complexation, Lane B: release (treated with pAsp) and Lane C: protection (treated with RNAse A at 37 ± 0.1 °C and pAsp). C = 1× RNAse A (45 units per μL), C_1 = 2.5× RNAse A, C_2: 5.0× RNAse A.

intact siRNA was also observed (Lane C). Furthermore, greater amounts of naked siRNA were degraded at higher concentrations of RNAse A (2.5 times the standard amount (Lane C_1) and 5 times the standard amount (Lane C_2)). This result could be due to the altered activity of the RNAse A enzyme reported in the presence of NaCl. It is therefore not possible to distinguish whether the persistence of intact siRNA bands following RNAse treatment arises from effective protection within the nanocomplexes and/or from partial inhibition of RNAse A activity in the presence of NaCl. Consequently, the contribution of electrolyte-mediated enzyme inhibition cannot be excluded. While high salt concentrations are reported to reduce the rate of catalysis of RNAse A,[44] interpretation at lower salt concentrations is further complicated by effects on low-level contaminants present in common buffers.[45] These factors together make it difficult to attribute siRNA stability solely to nanocomplex-mediated protection.

How do the cationic peptide sequence and electrolyte affect the complexation efficiency of siRNA within the lipopolyplexes?

Next, the efficiency of complexation of siRNA in the various LPRs was examined using a PicoGreen™ fluorescence assay. This exploits the fact that PicoGreen™, once bound to siRNA, exhibits greater than a 10^3 times increase in fluorescence. Hence, determining the extent of PicoGreen/siRNA binding by quantifying the increase in PicoGreen fluorescence, allows an estimation of the amount of unbound siRNA in the various LPR formulations.[46]

LPRs were prepared at different P : R charge ratios whilst keeping the L : R charge ratio constant at 0.5 : 1. The PicoGreen assay for the LPRs prepared fully in water (**VwLPRw**) is shown in Fig. 4a. For the **VwLPRw** prepared using the Group 1 peptides, less than 20% fluorescence was observed at a P : R charge ratio of 1 : 1 and no major change was observed at higher P : R charge ratios. However, for the VwLPRw prepared with **H_{12}BLY**, the apparent extent of complexation gradually increased upon increasing the P : R charge ratio up to 18 : 1. LPRs prepared using Group 2 peptides demonstrated a similar trend, but with a slightly lower level of complexation efficiency than the LPRs containing Group 1 peptides. These results are in apparent variance with the gel electrophoresis experiments, which implies that all of the siRNA is complexed by the LPR even for the **H_{12}BLY** sequence. However, we have previously hypothesised[25] that our method of preparation of lipopolyplexes results in the unentrapped nucleic acid cargo (pDNA or siRNA) being complexed with the cationic peptide. This would result in the siRNA : peptide complex remaining stationary in the agarose gel wells, preventing the siRNA from travelling down the gel: however, the peptide complexed siRNA is unavailable to interact with the PicoGreen.

For the LPRs prepared fully in 120 mM NaCl (**VsLPRs**) there are more striking differences (Fig. 4b). Specifically, Group 1 peptides **H_{12}BLY** and **K_4BLY** show a fluorescence around 50% at a P : R ratio of 1 : 1, whereas **K_{12}BLY** and **H_{12}BLY** appear more complexed at this ratio with <20% fluorescence observed. Interestingly, more **K_4BLY** was complexed in the LPRs made in water than in aqueous 120 mM NaCl. Group 2 peptides **$(HHR)_4$BLY**, **$(HR)_6$BLY** and **$(HK)_6$BLY** show a fluorescence around 50% at a P : R ratio of 1 : 1, whereas **$(RK)_6$BLY** appears more extensively complexed exhibiting a fluorescence of around 20% at this ratio, decreasing to about 10% at a peptide to siRNA ratio of 2 : 1. For the three remaining peptides, the extent of complexation gradually increased upon increasing the P : R charge ratio up to 18 : 1, with the fluorescence at or below 20% at higher charge ratios. More of **$(HHR)_4$BLY**, **$(HR)_6$BLY** and **$(HK)_6$BLY** were complexed in the LPRs made in water than in aqueous 120 mM NaCl. Considering the VsLPRs formulations, it is notable that the cationic peptides that give the best knockdown in media, **H_{12}BLY** and **$(HR)_6$BLY**, exhibit a lower level of complexation with siRNA. By contrast the formulations with extensive or complete siRNA complexation, particularly **R_{12}BLY**, **K_{12}BLY** and **$(RK)_6$BLY**, exhibit significantly poorer knockdown capacity in media.

What is the effect of peptide sequence and electrolyte on the size and structure of the lipopolyplexes?

The apparent hydrodynamic size and ζ-potential were determined by dynamic light scattering (DLS) and electrophoretic light scattering techniques respectively. Data for the vesicles (Vw) and LPRs prepared in water (VwLPRw) are shown in Fig. 5a. As we have previously observed,[27] the LPRs were small, with a size range of 40–70 nm and ζ-potential of the order of 30–42 mV. With the exception of the LPR formulated from

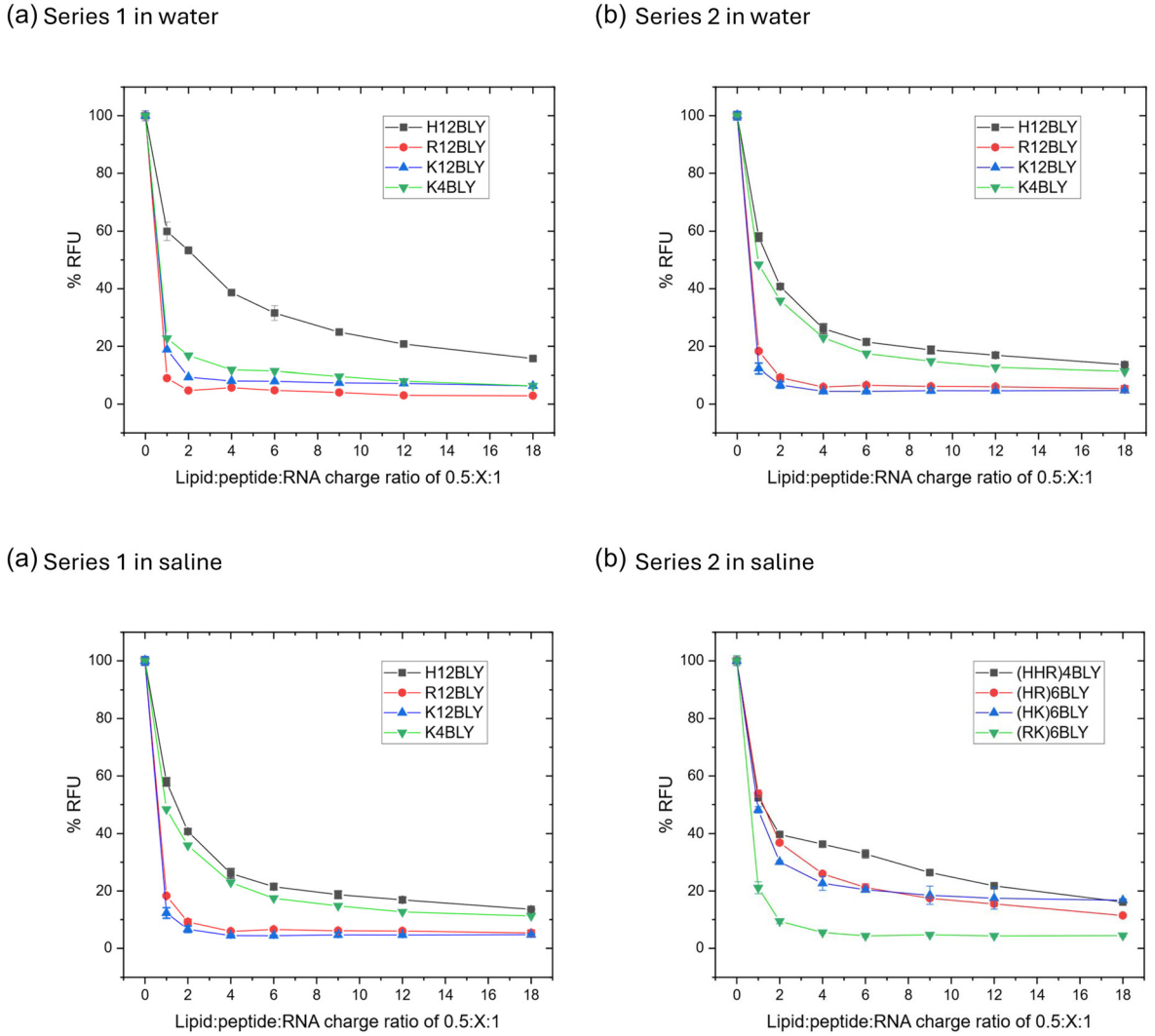


Fig. 4 Quantification of siRNA complexed in lipopolyplexes (LPRs) prepared (a) in water (VwLPRw) (LHS = Group 1 peptides and RHS Group 2 peptides) and (b) in 120 mM saline (VsLPRs) (LHS figures = Group 1 peptides and RHS figures = Group 2 peptides) determined as relative fluorescence units (RFU) using a picogreen fluorescence assay. LPRs containing 0.02 mg mL^{-1} of (–)siRNA and an LR ratio of 0.12 : 1 Error bars are the SD of three measurements (n = 3) at 25 ± 0.1 °C. In most instances, the error bars are contained within the symbols.

K_4BLY (~70 nm) there were no significant differences in the apparent hydrodynamic size and the ζ-potential of the LPRs prepared at the two charge ratios and with the two series of peptides. We also determined the polydispersity index (PDI) : LPR can be considered to be relatively homogeneous if the PDI is less than 0.1 or 0.15 at most. Interestingly the parent vesicles were the most heterogeneous of the preparations, exhibiting a PDI of ~0.30. The LPRs were all heterogeneous as they exhibited PDIs in the approximate range 0.20–0.275.

To establish the effect of the presence of NaCl on the biophysical properties and knockdown efficacy of the LPRs, we also determined the apparent hydrodynamic size and ζ-potential of the vesicles and VsLPRs (Fig. 5b). The apparent hydrodynamic size of the cationic vesicles (Vs) prepared in 120 mM NaCl solution were ~ 3 times larger than those prepared in water (Vw), suggesting they are multilamellar in nature. Furthermore, the mean ζ-potentials measured for Vs were slightly lower (namely 32.5 mV) when compared to the corresponding values obtained for Vw (namely 42.5 mV), most probably due to the presence of the Cl ions reducing the positive charge on the cationic vesicles.

The mean apparent hydrodynamic size of the VsLPRs were also significantly larger than the VwLPRw prepared in the present study and those observed previously[27] complexing DNA as opposed to siRNA. VsLPRs prepared at the lower L : P : R charge ratio of 0.5 : 6 : 1 ranged in mean apparent hydrodynamic size from almost 200–275 nm. At the higher charge ratio of 0.5 : 12 : 1 the VsLPRs ranged in mean apparent hydrodynamic size from ~ 175–375 nm, with LPRs formulated from **R_{12}BLY**, **$(HHR)_4$BLY**, **$(HR)_6$BLY** and **$(HK)_6$BLY** being appreciably larger. The VsLPRs were also larger than the Vs from which they were prepared.

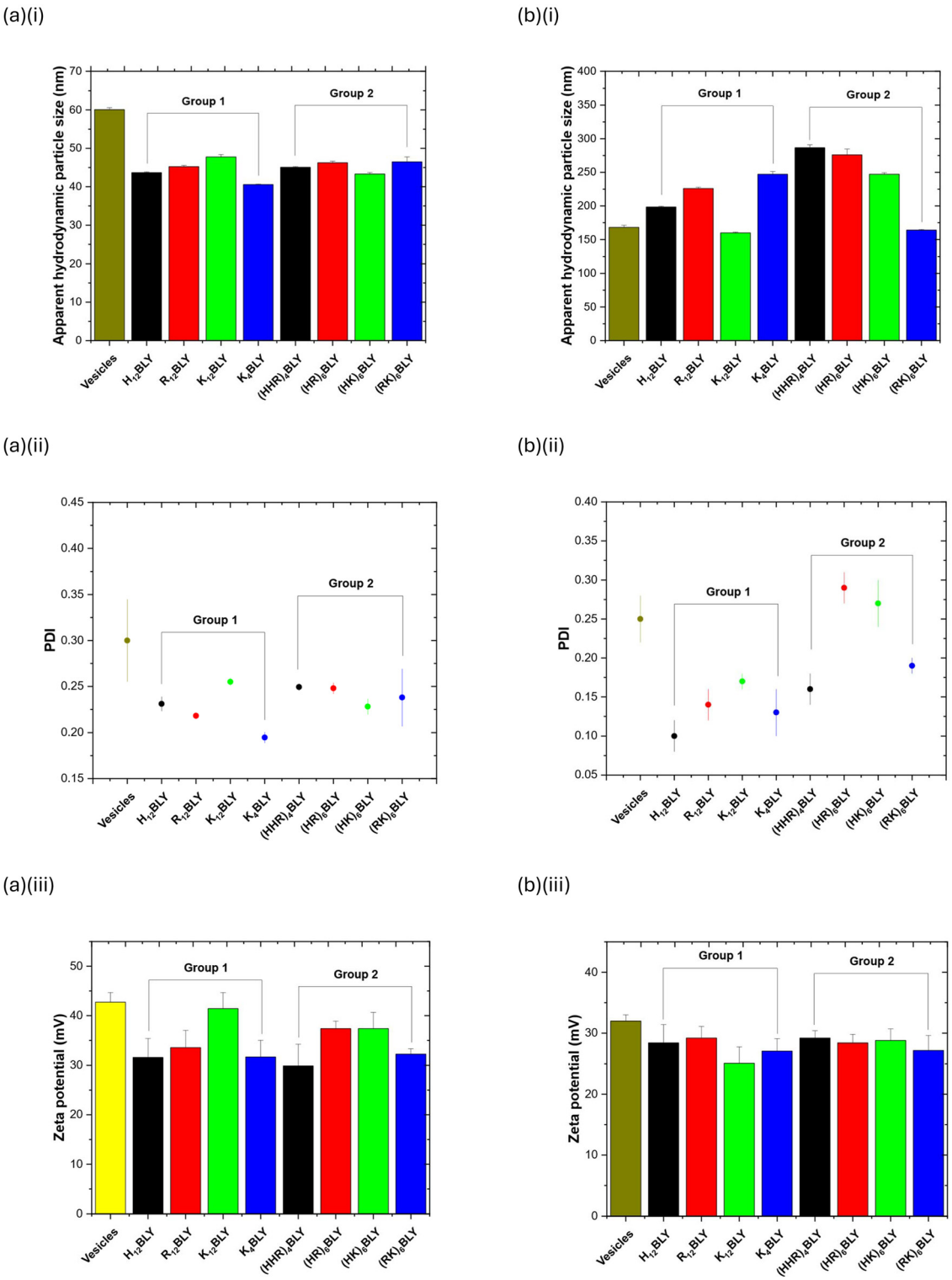


Fig. 5 The variation in (i) apparent hydrodynamic size, (ii) polydispersity index (PDI) and (iii) ζ-potential of lipopolyplexes (LPRs) made in (a) water and (b) 120 mM NaCl solution (VsLPRs). LPRs prepared at L : P : R charge ratio of 0.5 : 12 : 1 using 0.025 mg mL^{-1} of (–)siRNA. Cationic vesicles composed of DOTMA/DOPE at 1 : 1 molar ratio. Error bars are SD of three measurements (n = 3) at 25 ± 0.1 °C.

Although the mean ζ-potentials measured for the VwLPRw were very slightly higher (in the range 27–42 mV) when compared to the corresponding values obtained for VsLPRs (in the range 25–35 mV), there was no significant difference in the mean ζ-potential of the VwLPRw and VsLPRs prepared from the Group 1 and Group 2 peptides and at the two charge ratios

(Fig. 5 and Fig. S5). Interestingly, the VsLPRs made at the 0.5 : 12 : 1 charge ratio were relatively polydisperse, with the majority of the PDI at 0.20 or greater (Fig. 5a(ii)).

The stability of the LPRs over a period of 1 week were also studied. All Vw and VwLPRw formulations were stable with respect to their mean apparent hydrodynamic size at around 60 nm (Fig. S6a(i)), while the PDI also remained stable at ~0.25 for about half of the LPR types (Fig. S6b(ii)), the other half exhibited an increase in PDI after 3–5 days storage. In comparison, the mean apparent hydrodynamic size of the Vs and VsLPRs prepared in 150 and 120 mM NaCl solution, respectively increased from between 150–300 nm to several hundred nm over the course of a 1 week (Fig. S6b(i)) with a corresponding increase in the PDI (Fig. S6b(ii)) with the LPRs prepared containing **H_{12}BLY** and the Group 2 peptides exhibited the least increase in mean apparent hydrodynamic size. This result suggests that the LPR prepared in saline solution are less stable than those prepared in water, which is probably a consequence of the slightly reduced charge present on the surface of the Vs and VsLPRs in the presence of NaCl.

From a translational perspective, the relatively large size of the VsLPRs (*ca.* 175–375 nm) and their reduced colloidal stability are important considerations. Nanoparticles in this size range remain compatible with endocytic uptake pathways, although efficiency and intracellular trafficking are strongly size- and cell-type-dependent. The high levels of gene silencing observed in A549 cells in Fig. 2 suggest that VsLPRs are effectively internalised despite their larger size.

However, the increase in particle size and reduced stability over time may present challenges for *in vivo* applications, where prolonged circulation and reproducible dosing are required. Approaches such as PEGylation or lyophilisation could improve stability, but any such strategies would need to preserve the multilamellar and structural properties observed, in order to maintain activity. This therefore remains an area for further investigation.

To determine the effect that the presence of NaCl has on the self-assembly and structure of the vesicles and LPRs, a series of small angle neutron scattering (SANS) experiments were performed. Since the structure of the DOTMA : DOPE vesicles used to prepare the LPRs in the absence and presence of increasing amount of NaCl have been reported previously[25] these data are not discussed in detail here. Reassuringly, the trend of the results obtained here (Table S1) were similar to those reported earlier[25] in that as the amount of NaCl present increased, the structure of the vesicles became increasingly multilamellar. Furthermore, the vesicle's bilayer thickness decreased upon increasing NaCl concentration, from 43.6 ± 0.4 Å in the absence of NaCl (Vw) to 37.9 ± 0.1 Å in the presence of 80 mM NaCl (Vs), while the *d*-spacing increased to 78 ± 1 Å, most likely due to the slight reduction in the charge of the vesicles in the presence of increased NaCl.

Corresponding SANS experiments performed on VwLPRw and VsLPRs investigated the effect of NaCl on the LPR self-assembly and structure. The peptide **$(HHR)_4$BLY** was selected as a representative sequence, as VsLPRs with this peptide show intermediate levels of knockdown (Fig. 2) and also allowed a comparison with SANS studies carried out on the VsLPDs studied in our previous work with DNA which were formulated with **$(HHR)_4$BLY**.[25] Table S2 shows that the thickness of the DOTMA : DOPE bilayers in the **$(HHR)_4$BLY** LPRs prepared at a L : P : R charge ratio of 0.5 : 6 : 1 were generally comparable to those measured for the parent vesicles. Moreover, the *d*-spacing and amount of multilamellar LPRs also increased with NaCl concentration, with 100% of the vesicles being multilamellar at 80 and 120 mM NaCl.

To ensure that the results obtained for the LPRs containing **$(HHR)_4$BLY** were representative of LPRs containing the other peptides studied, LPRs prepared in the absence and presence of 120 mM NaCl and containing peptides from both Groups were examined at a L : P : R charge ratio of 0.5 : 12 : 1 (Table 1 and Fig. S7). The corresponding data obtained for LPRs prepared at a L : P : R charge ratio of 0.5 : 6 : 1 in water are shown in Table S3 and Fig. S8. The bilayer thicknesses obtained for the various LPRs when prepared in water and in the presence of NaCl were generally comparable to those reported for **$(HHR)_4$BLY**-containing LPRs. Furthermore, the LPRs dispersed in water have bilayers of *ca.* 41–43 Å thick, whereas those prepared in 120 mM NaCl solution are (in almost all cases) *ca.* 2–3 Å thinner. Additionally, consideration of the Lorentz factor used to fit the LPRs, shows that the LPRs prepared in saline seem, in most cases, to be more rigid and exhibit less variability in their local surface curvature. In the absence of NaCl the LPRs contain only one bilayer while in the presence of 120 mM NaCl there is an increase in the lamellarity of the LPRs. This is independent of peptide type and, where examined, L : P : R ratio. A similar result was previously seen for the LPDs.[25] It is worth commenting that while the *d*-spacings

Table 1 Structural parameters obtained from model fitting the SANS data obtained for LPRs prepared at a lipid : peptide : siRNA ratio of 0.5 : 12 : 1 from DOTMA : DOPE vesicles at a 1 : 1 molar ratio and containing the Group I and Group II peptides and siRNA and dispersed either in D_2O or 120 mM NaCl solution was measured at 25 ± 0.1 °C. Figures in brackets indicate the number of bilayers present in the LPR

L : P : R	0.5 : 12 : 1		
Peptide	Bilayer thickness	% lamellarity	*d*-Spacing $\sigma(d)$/d
D_2O			
K_{12}BLY	42.2 ± 0.5	(1) 100	—
R_{12}BLY	42.8 ± 0.5	(1) 100	—
H_{12}BLY	42.7 ± 0.5	(1) 100	—
$(HR)_6$BLY	43.1 ± 0.5	(1) 100	—
$(HK)_6$BLY	42.0 ± 0.5	(1) 100	—
HHR_4BLY	N/D		
120 mM NaCl			
K_{12}BLY	N/D		
R_{12}BLY	34.9 ± 4.5	(2) 14 (3) 86	194 ± 6/0.4
H_{12}BLY	39.0 ± 0.1	(1) 55 (5) 45	102 ± 1/0.3
$(HR)_6$BLY	38.8 ± 1.9	(1) 49 (5) 51	101 ± 1/0.3
$(HK)_6$BLY	42.6 ± 4.4	(3) 100	158 ± 0.5
HHR_4BLY	34.9 ± 4.5	(1) 46 (5) 54	224 ± 19/0.4

N/D = not determined.

obtained for the LPRs were large the precise value varied, although the reason for this difference is currently not known.

Discussion

We have shown that formulating siRNA into LPR in NaCl solution results in nanocomplexes with greatly enhanced levels of luciferase gene silencing in serum. Outstanding results were seen when the LPRs were formulated using either **H_{12}BLY** or **$(HR)_6$BLY**. With these two cationic peptides strikingly high (>80%) levels of knockdown were observed: these LPR formulations had better activity than the Lipofectamine 2000-siRNA control but without the toxicity usually observed with Lipofectamine.

Some enhancement in knockdown levels was also observed when the LPRs were formulated using **$(HHR)_4$BLY**, **$(HK)_6$BLY** or **K_4BLY**, but little or no improvements were observed with LPRs formulated in saline using **K_{12}BLY**, **$(RK)_6$BLY** or **R_{12}BLY** (Fig. 2b).

All knockdown experiments in the present study were carried out in A549 cells, using a targeting peptide sequence designed to bind human airway epithelial cells. Further testing is required to establish the broader applicability of the NaCl-based formulations in other cell types.

Comparing the LPR complexes formulated in saline (VsLPRs) with those formulated in water (VwLPRw), it is clear that the VwLPRw formulations lead to small, compact nanocomplexes which are similar in physicochemical properties to the VwLPRw formulations that we previously reported[27] with different cationic peptides. However, when formulated in saline both the SANS and DLS data indicate that all of the VsLPRs are significantly bigger, regardless of cationic peptide sequence, although no difference in surface charge was observed. The larger size of the VsLPRs is accompanied by a concomitant increase in lamellarity and *d*-spacing, which also increases with increased concentration of NaCl. We also observed this multilamellarity and increased *d*-spacing when the precursor vesicles were formulated in saline (Vs: Table 1) and in our previous work with VsLPDs.[25] Although these larger, multilamellar nanocomplexes are also less stable over a week than their VwLPRw counterparts (Fig. S6) they all appear to protect the siRNA cargo against RNAse degradation (Fig. 3). However, it is not possible to unambiguously conclude that the siRNA was protected from RNAse A when entrapped inside the various VsLPRs, because the NaCl may be altering the activity of the RNAse A. This is in line with our previous work[25] with VsLPDs, where we also observed that protection against pDNA degradation by DNAse I increases with increased concentration of NaCl, and increasing concentration of NaCl also appears to impede the action of DNAse I. From consideration of these results, it is likely that the peptide/siRNA complex is present in the core of the LPRs, with only a small amount of complex being present in the water layers trapped between the cationic lipid bilayers in those LPRs made fully in the presence of NaCl (VsLPRs). This most likely accounts for the high polydispersity observed in the *d*-spacing observed with the SANS experiments.

Previous studies have demonstrated that lipoplexes formulated with NaCl give optimised nanocomplexes for delivery of pDNA[47–49] and siRNA.[50] In addition to its effects on nanocomplex structure, NaCl may also influence interactions with serum proteins. In physiological media, negatively charged proteins such as BSA can adsorb onto cationic nanoparticles and affect their stability and cellular uptake. Increased ionic strength may partially screen these interactions, potentially altering protein corona formation and nanoparticle behaviour. These effects may contribute to the improved knockdown observed in serum, although this was not directly investigated here and represents an important area for future study.

Significantly, these formulations led to large (>700 nm), multilamellar, colloidally unstable complexes which induced efficient transfection of the nucleic acid cargo. This was attributed to a combination of factors including favourable size for cellular uptake, greater surface charge and/or charge screening by the electrolyte. To our knowledge this is the first time that a similar enhancement of siRNA delivery by formulating lipopolyplexes in NaCl has been observed and studied.

However, all of these characteristics of the VsLPRs appear similar or the same regardless of which cationic peptide sequence was used. In order to understand the enhanced levels of VsLPRs knockdown achieved with **H_{12}BLY** or **$(HR)_6$BLY** (and the low knockdown achieved with **K_{12}BLY**, **$(RK)_6$BLY** and **R_{12}BLY**) we have studied the level of siRNA complexation, as observed from the PicoGreen measurements. Here, there is a clear difference between formulations. Where the siRNA is more loosely/partially complexed (**H_{12}BLY**, **$(HR)_6$BLY**, **$(HHR)_4$BLY** and **$(HK)_6$BLY**) good or excellent levels of gene silencing are observed. By contrast, formulations with higher levels of siRNA complexation (**R_{12}BLY**, **K_{12}BLY** and **$(RK)_6$BLY**) have reduced or no knockdown capacity. We have previously shown[25] that the apparent discrepancy between the gel electrophoresis results (which appear to show complete complexation in all cases) and the PicoGreen studies is likely due to the fact that the PicoGreen can also detect the nucleic acid cargo in the looser peptide : nucleic acid complexes found in the NaCl formulations, and that the presence of NaCl does not affect the accuracy of the PicoGreen experiments.

As we have shown that the macromolecular architecture of all of these VsLPRs are similar, the beneficial effects of the looser complexation of the siRNA cargo with the His-containing sequences (**H_{12}BLY**, **$(HR)_6$BLY**, **$(HHR)_4$BLY** and **$(HK)_6$BLY**) must arise from siRNA : peptide interactions at the molecular level. An important question is the relative contribution of ionic strength and peptide–siRNA interactions to the enhanced knockdown efficiency. While NaCl plays a key role in promoting the formation of larger, multilamellar structures, these features are common across all VsLPRs, including those with poor activity. In contrast, the degree of siRNA complexation (determined by the PicoGreen assay) correlates more directly with biological activity, with the most effective formulations exhibiting relatively looser binding. Together, these findings suggest that NaCl primarily governs the lipopolyplex lamellarity, while peptide–siRNA interactions more strongly influence

cytoplasmic release and gene silencing efficiency, with both factors contributing to optimal activity.

The complexation of His/Arg-rich peptides with siRNA has not been extensively studied. Arg-based polyplexes are increasingly important as nucleic acid delivery vectors, and this has been variously attributed to the propensity of Arg side chains to alter membrane curvature, mediate cell-penetration and to form bidentate hydrogen bonding interactions with the phosphate backbone.[33] Recent NMR and molecular dynamics studies[51,52] have shown that guanidine-guanidine interactions rigidify Arg-containing cationic peptides and their interactions with nucleic acids. Furthermore, in His/Arg cationic peptides, the Arg side chains modulate the pK_a of the His side chains,[53] resulting in a hydrogen-bonding network which varies with pH.[54] These factors will likely influence the loose complexation between the **H_{12}BLY** and **$(HR)_6$BLY** peptides and their siRNA cargo in the presence of NaCl, and may allow the cargo to be released more efficiently into the cytoplasm after internalisation of the lipopolyplexes. It should be noted that the proposed link between looser siRNA complexation (as indicated by PicoGreen studies) and enhanced cytoplasmic delivery remains correlative in the present study. While the improved gene silencing observed is consistent with more effective intracellular delivery, direct visualisation of cytoplasmic release for these formulations would be required to confirm this.

Conclusions

In this work we have demonstrated that formulating siRNA-based lipopolyplexes in the presence of NaCl gives formulations that are highly effective in gene silencing in the presence of serum. We have carried out a detailed structural and biophysical investigation of these VsLPRs and have shown that they are large, multilamellar complexes which efficiently encapsulate and protect siRNA. Particularly exciting results were obtained when His or His/Arg cationic peptides, **H_{12}BLY** and **$(HR)_6$BLY**, were used in the formulations. These VsLPRs give excellent levels of gene knockdown in serum which exceed those obtained with the "industry standard" Lipofectamine. The key factor influencing these high levels of activity appears to be a looser complexation between the cationic peptide and the siRNA. Further work is needed to determine the nature of the interactions between His and Arg side chains, NaCl, and the negatively charged siRNA phosphate backbone and to elucidate why this arrangement is particularly favourable for delivery of siRNA. This will enable the design of LPR complexes that can be used for highly effective delivery of therapeutic siRNA under physiologically relevant conditions, paving the way for next generation advanced RNA-based therapies with reduced toxicity.

Author contributions

The peptide synthesis was carried out by LC and FC. LPR formulation and characterisation, knockdown, gel retardation and PicoGreen assays were carried out by LC and LK. SANS measurements were carried out by LC, YM, AET, NM and MJL, and the data analysed by DJB and MJL. The project conceptualisation was by all authors and the project was supervised by MJL, ABT, HCH and LK. The original draft of the paper was written by ABT and MJL, and the manuscript has been reviewed and edited by all contributing authors.

Conflicts of interest

There are no conflicts to declare.

Data availability

The data supporting this article have been included as part of the supplementary information (SI). Supplementary information: luciferase gene silencing activities, measurements of hydrodynamic size and polydispersity, SANS data. See DOI: https://doi.org/10.1039/d6pm00042h.

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