

Lipid-mediated DNA and siRNA transfection efficiency depends on peptide headgroup†

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A series of amphiphiles with differing cationic tri- and di-peptide headgroups, designed and synthesized based on lysine (**K**), ornithine (**O**), arginine (**R**), and glycine (**G**), have been characterized and evaluated for DNA and siRNA delivery. DNA–lipoplexes formed from the tri- and di-lipopeptides possessed lipid : nucleic acid charge ratios of 7 : 1 to 10 : 1, diameters of ~200 nm to 375 nm, zeta potentials of 23 mV to 41 mV, melting temperatures of 12 °C to 46 °C, and lamellar repeat periods of 6 nm to 8 nm. These lipid–DNA complexes formed supramolecular structures in which DNA is entrapped at the surface between multilamellar liposomal vesicles. Compared to their DNA counterparts, siRNA–lipoplexes formed slightly larger complexes (348 nm to 424 nm) and required higher charge ratios to form stable structures. Additionally, it was observed that lipids with multivalent, tripeptide headgroups (*i.e.*, **KGG**, **OGG**, and **RGG**) were successful at transfecting DNA *in vitro*, whereas DNA transfection with the dipeptide lipids proved ineffective. Cellular uptake of DNA was more effective with the **KGG** compared to the **KG** lipopeptide. In siRNA knockdown experiments, both tri- and di-peptide lipids (*i.e.*, **RGG**, **GGG**, **KG**, **OG**, **RG**, **GG**) showed some efficacy, but total cellular uptake of siRNA complexes was not indicative of knockdown outcomes and suggested that the intracellular fate of lipoplexes may be a factor. Overall, this lipopeptide study expands the library of efficient DNA transfection vectors available for use, introduces new vectors for siRNA delivery, and begins to address the structure–activity relationships which influence delivery and transfection efficacy.

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1 Introduction

The delivery of therapeutic nucleic acids to correct a defective gene, introduce a new gene, or knockdown a gene is of intense interest. Although polymers,^{1–3} dendrimers,^{4–6} inorganic nanoparticles,^{7–9} and their hybrids are commonly used for gene delivery, lipids are still the most intensively studied among all synthetic vectors,^{10–17} including for use in clinical trials. Because of the relatively large size and negative charge of the nucleic acid, the first step of delivery involves the complexation between the vector and nucleic acid. In most cases, this complexation is governed by electrostatic interactions between the positively charged vectors and the negatively charged phosphate backbone of the nucleic acid, as well as the hydrophobic interactions between the individual assembled amphiphiles. A variety of

amines have been incorporated into the lipid structure for nucleic acid binding, including nature-inspired molecules or structures. For instance, spermine, imidazole (or histidine), and nucleoside (or nucleotide) headgroup derivatives have been extensively investigated.^{18–24} Additionally, molecules such as cell penetrating peptides (CPPs) and cholesterol derivative have been added to improve gene delivery.^{25–30}

Recently, our group reported the use of tripeptides as the DNA binding domains in amphiphilic lipids for gene delivery.^{31,32} These lipopeptides were based on known nucleic acid binding peptides and exhibited high transfection efficiency *in vitro*, comparable to the widely used commercial transfection agent Lipofectamine 2000 (Invitrogen).³¹ Additionally, it was found that amphiphiles with amino acid linkers in the headgroups outperformed those with alkyl or ethylene oxide linkers, where the latter only showed minimal transfection activity.³² Herein we describe the synthesis, physicochemical properties, cytotoxicity, and *in vitro* gene transfection activity of a series of new lipopeptides based on lysine (**K**), ornithine (**O**), arginine (**R**), and glycine (**G**) (Fig. 1). By changing the terminal cationic amino acid (**K**, **O**, **R**, and **G**) while varying the spacing group from glycine to glycine–glycine (**GG**), the effect of the lipid composition on DNA and siRNA transfection efficiency can be evaluated.

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2 Material and methods

Please see the ESI† document for complete details on the synthesis of the eight lipopeptides, the characterization studies, and the *in vitro* experiments.

3 Results and discussion

The motivation for these studies stems from the hypothesis that lipopeptides possessing either tri- or di-peptide headgroups should be effective for DNA and siRNA transfection with the dicationic tripeptides being more efficient than the dipeptides because they possess two positive charges per lipid and will form a more stable lipoplex. The lipopeptides were synthesized following methods similar to those described previously.³¹ The headgroup of these lipopeptides are either tri- or di-peptides with **GG** or **G** as the spacing group between the cationic **K**, **O**, **R**, or **G** terminal group and the hydrophobic domain. All the lipopeptides have the same glycerol backbone and two C14:0 hydrophobic chains. Therefore, the **KGG**, **OGG**, **RGG**, **KG**, **OG**, and **RG** possess 2 positive charges per molecule; while the **GGG** and **GG** analogs possess 1 positive charge per molecule under physiological conditions. The synthetic approach to these lipids involved first preparing the glycerol backbone containing the alkyl ester chains, followed by installation of the peptide headgroup. The complete synthesis protocols and experimental details can be found in the ESI.†

The physicochemical characteristics of the liposomes and lipoplexes were studied using a variety of techniques, including binding assays, melting temperature measurements, and X-ray structural analysis. For these studies liposomes were prepared by a film hydration method, followed by sonication and extrusion to produce monodisperse liposome particles. The liposomes were then diluted and mixed with DNA to induce complexation, forming the final lipoplexes. Detailed descriptions of particle formation, for the various experiments, are found in the ESI.† First, a standard ethidium bromide (EtBr)–DNA displacement fluorescence quenching assay³³ was performed to determine the DNA-binding capability of the lipopeptides. Fig. 2 shows that the fluorescence signal decreased as a function of lipopeptide–DNA (+/–) charge ratio. The

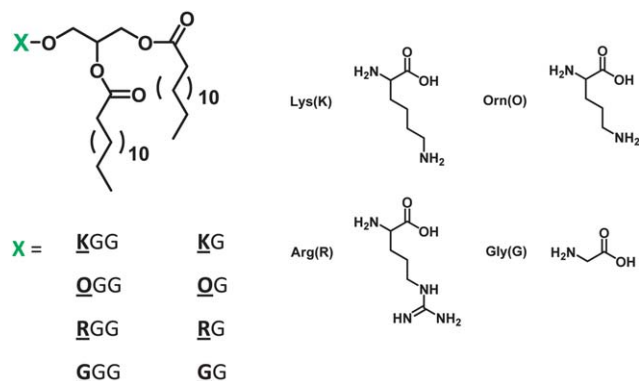


Fig. 1 Library of lipopeptides with tri- or di-peptide headgroups.

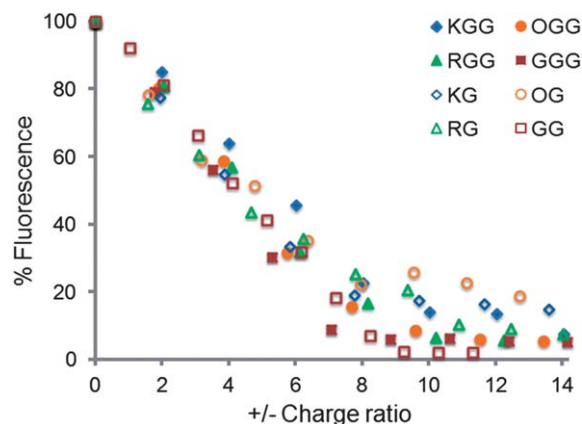


Fig. 2 EtBr–DNA displacement assay showing the fluorescence intensity as a function of lipopeptide–DNA (+/–) charge ratio.

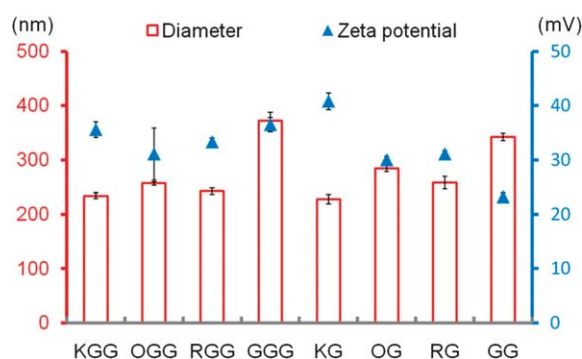


Fig. 3 Diameters and zeta potentials of peptide-based lipoplexes.

maximum EtBr displacement was achieved at charge ratios between 8 : 1 and 12 : 1 for all of the tri- and di-peptide lipids. This suggests that the DNA binding affinities are similar for the lipopeptides regardless of the spacing group (**GG** vs. **G**), the composition of the positive charge (**K** vs. **R**), the size of the headgroup (**K** vs. **G**), or the total charge (2+ vs. 1+). Next, the diameters of the lipoplexes were measured by dynamic light scattering (DLS) at a lipopeptide–DNA charge ratio of 8 : 1. The results in Fig. 3 show that all the tri- and di-peptide lipoplexes were similar in size (between 200 nm and 300 nm), except the lipoplexes of **GGG** and **GG** which were slightly larger in size (~375 nm). The **GGG** and **GG** lipoplexes tended to aggregate more in solution than the other lipoplexes. The zeta potentials of the various lipoplexes ranged from 23 mV to 41 mV, with the **GG** lipopeptide possessing the lowest zeta potential (Fig. 3). There were no systematic differences between the tri- and di-peptide lipids in these studies.

Differential scanning calorimetry (DSC) experiments were performed to evaluate the temperature at which the lipid membrane transitions from the gel state to the liquid-crystalline state for the various amphiphiles. Data on the lipopeptides, as shown in Table 1, was collected in the presence and absence of DNA and informs us of the lipid state at 37 °C when the lipid will be in contact with the cellular membrane. In the absence of DNA the lipopeptides with **K**, **O**, and **R** terminal headgroups had

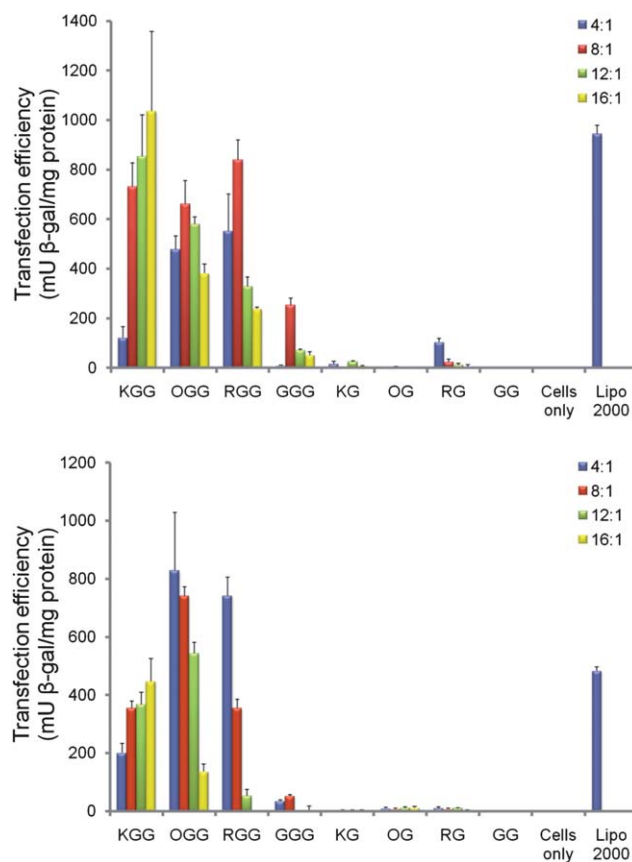
Table 1 Melting temperatures (T_m) and repeat periods for lipopeptides in the presence and absence of DNA

Amphiphile	T_m ($^{\circ}\text{C}$)		Repeat period (nm)	
	No DNA	With DNA	No DNA	With DNA
KGG	15.7	11.8	6.0	6.6
OGG	15.1	14.0	8.2	8.1
RGG	14.8	14.2	6.7	6.9
GGG	30.4	32.5	5.7	6.2
KG	18.9	16.5	6.1	6.6
OG	16.9	14.9	6.0	6.1
RG	15.2	15.1	5.9	6.1
GG	34.6	46.0	5.5	5.9

similar melting temperatures, T_m , values (ranging from 14.8 $^{\circ}\text{C}$ to 18.9 $^{\circ}\text{C}$). The addition of DNA resulted in similar and slightly lower T_m values for these lipids (11.8 $^{\circ}\text{C}$ to 16.5 $^{\circ}\text{C}$). Liposomes and lipoplexes formed from these lipopeptides will have fluid liquid-crystalline membranes *in vitro*, potentially increasing the likelihood of interaction with the cellular membrane and delivery of the DNA payload to affect transfection. On the other hand, **GGG** and **GG** showed melting temperatures approximately 15 $^{\circ}\text{C}$ higher (30.4 $^{\circ}\text{C}$ and 34.6 $^{\circ}\text{C}$, respectively) than observed for the other lipopeptides. This is likely a result of the smaller headgroup and single cationic charge of **G**; hence, the smaller steric and repulsive forces among neighboring molecules result in more compact packing in the supramolecular structure. It is important to note that this trend was also observed in the presence of DNA (Table 1).

X-ray diffraction (XRD) was performed to characterize the structure formed by these lipopeptide amphiphiles in the presence or absence of DNA. Although no discrete X-ray reflections were observed for fully hydrated specimens, multiple reflections indexing as orders of lamellar repeat periods were observed after partial dehydration in a controlled relative humidity atmosphere that removes water from between adjacent bilayers without removing water from within the lipid headgroup region.³⁴ Such lamellar repeat periods are characteristic of lipid bilayers. In the presence or absence of DNA the repeat periods ranged from 5.5 nm to 6.9 nm, except for **OGG** (Table 1). The reason that **OGG** had such large repeat periods is not known, but clearly a result of **O** as opposed to **G**, **K**, or **R**. For each lipopeptide the repeat period changed only a few tenths of a nanometer in the presence of DNA. Since the diameter of one monolayer of B-DNA, including a hydration shell, is ~ 2 nm,^{35,36} the slight increase of repeat period is not enough to accommodate DNA. Therefore, the data are consistent with a structural model where the DNA is entrapped at the interface between multilamellar liposomes in solution.³⁷ This lipoplex structure is different than the complexes formed from DNA with either DOTAP³⁵ or cationic triesters of phosphatidylcholine,³⁸ where a smectic phase is formed with the DNA located between the adjacent lipid bilayers within the multilamellar liposome.

Transfection experiments using the reporter gene β -galactosidase (β -gal, Promega) were performed with Chinese hamster ovarian (CHO) and mouse embryonic fibroblast (NIH3T3) cells

**Fig. 4** DNA transfection after 48 hours in CHO (top) and NIH3T3 (bottom) cells as a function of lipopeptide–DNA molar ratio. Lipofectamine 2000 was used as a positive control.

(see ESI† for experimental details). Gene transfection results were determined after 48 h as a function of both lipopeptide composition and lipopeptide–DNA molar ratio (Fig. 4). In both cell lines, only the tripeptide lipids showed significant transfection activities, whereas the dipeptide lipids had very minimal activities. In general, lipopeptide–DNA molar ratios of 8 : 1 or 12 : 1 performed the greatest, with the difference being minimal between the best performing composition and the next best. The highest transfection efficiencies obtained in both cells are similar to the positive control, Lipofectamine 2000. Among the tripeptide lipids, **GGG** afforded the least activity, indicating the important role of the multi-charged headgroup in this system. Viability assays performed with the lipopeptides showed minimal cytotoxicity (viability > 80%) at the lipopeptide–DNA molar ratios that showed high transfection efficiency (Fig. 5). The **KGG** had increased cytotoxicity in CHO when used at molar ratios greater than 8 : 1, similarly **RGG** reduced viability (<80%) at all ratios tested.

It has been reported that the introduction of groups capable of hydrogen bonding to neighboring molecules in lipid headgroups can increase transfection activity; specifically, incorporation of a hydroxyethyl group in DOTMA and DOTAP showed improved DNA delivery efficiency.^{39,40} The headgroup hydration is decreased by incorporation of a hydroxyalkyl chain capable of hydrogen bonding to neighboring headgroups; consequently,

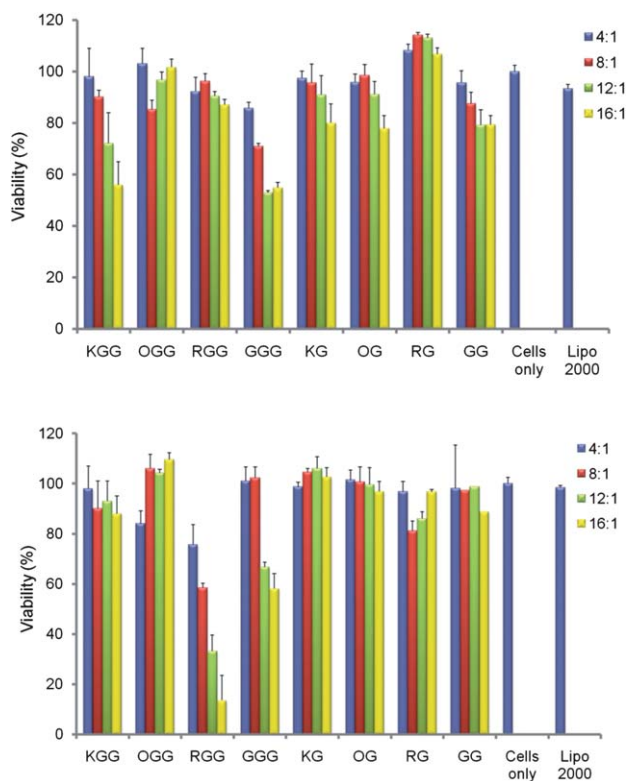


Fig. 5 Viability of lipopeptides in CHO (top) and NIH3T3 (bottom) cells as a function of lipopeptide–DNA molar ratio. Lipofectamine 2000 was used as a positive control.

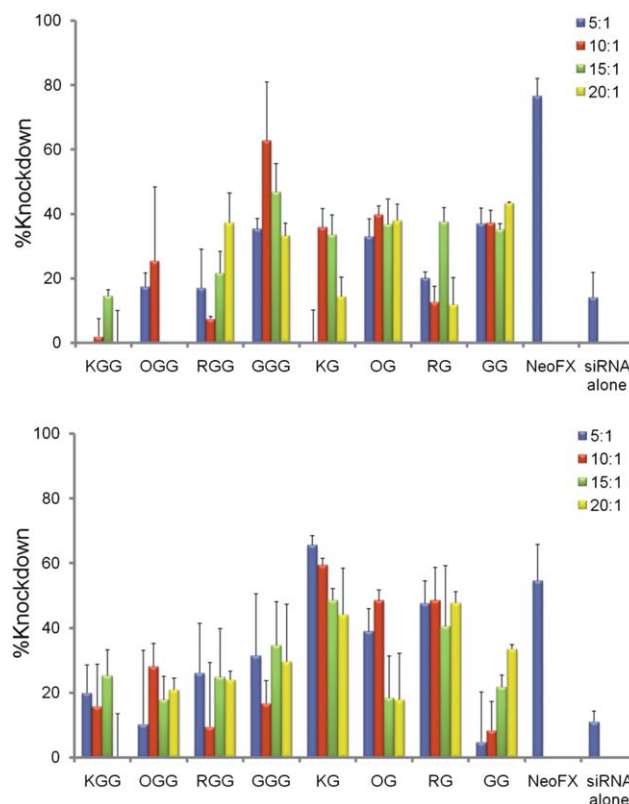


Fig. 6 GAPDH knockdown after 48 hours in CHO (top) and HepG2 (bottom) cells as a function of lipopeptide–siRNA molar ratio. NeoFX was used as positive control.

the cross-sectional area of the headgroup is reduced. This imbalance between the cross-sectional area of the headgroup and the hydrophobic, cone-shaped structure formed by the cationic lipid chains results in a more unstable lipid assembly. This supramolecular conformation enhances the likelihood that the lipoplex will undergo fusion with anionic vesicles. It is also known that lipoplex instability can lead to improved transfection, since fusion between the cationic lipoplex and the endosomal membrane leads to DNA release into the cytoplasm.^{41–44} In our case, the extra glycine in the tripeptide lipid likely results in less hydration in the headgroup, thus decreasing the lipoplex stability and leading to enhanced membrane fusion and improved transfection. However, the differences in DNA transfection between tri- and di-peptide lipids are so dramatic that this is probably only part of the underlying cause. This point will be revisited later in the manuscript.

siRNA transfections were also performed with all the lipopeptides using the KAlert GAPDH assay (Ambion) with CHO and human hepatocellular carcinoma (HepG2) cells (Fig. 6) at four different amphiphile–siRNA molar ratios (see ESI† for experimental details). NeoFX was used as the positive control. The GAPDH knockdown was measured 48 h after transfection. In contrast to DNA transfection, where only tripeptide lipids showed significant transfection activity, an entirely different trend was observed for siRNA knockdown. All the lipopeptides were capable of mediating the GAPDH knockdown to some

extent. The highest level of gene silencing, which was comparable to the positive control, was achieved by GGG in CHO cells and KG in HepG2 cells. In some cases, the dipeptide lipid performed even better than the respective tripeptide analog; for example, KG showed higher GAPDH knockdown than KGG in both cell lines.

The studies above reveal that the tri- and di-peptide lipids have different efficiency for DNA and siRNA transfection *in vitro*. Although DNA and siRNA are both nucleic acids and have anionic phosphodiester backbones with identical negative charge : nucleotide ratios, many differences exist between cationic lipid-mediated DNA and siRNA transfection. A fundamental difference is the mechanism of action. The DNA must be transferred into the nucleus to achieve gene expression, and nuclear entry has been identified as one of the major barriers in DNA delivery, whereas siRNA only needs to reach the cytoplasm to achieve gene silencing. Another difference is the mechanism of complexation of cationic lipids with either DNA or siRNA. Condensation occurs when liposomes bind to DNA and form a lipoplex, since DNA is a large and “flexible” macromolecule.⁴⁵ This condensation will only occur when the DNA is ~400 nucleotides in length or larger.⁴⁶ In comparison, siRNA is much smaller (~20 nucleotides) and more “rigid”, thus requiring larger charge ratios to overcome the free energy factors that often make lipid–siRNA complexes unstable.⁴⁷ This difference in interaction may consequently affect the complexation properties, cellular uptake, and trafficking.

Table 2 Comparison of physicochemical properties of lipoplexes formed by **KGG** and **KG** with siRNA

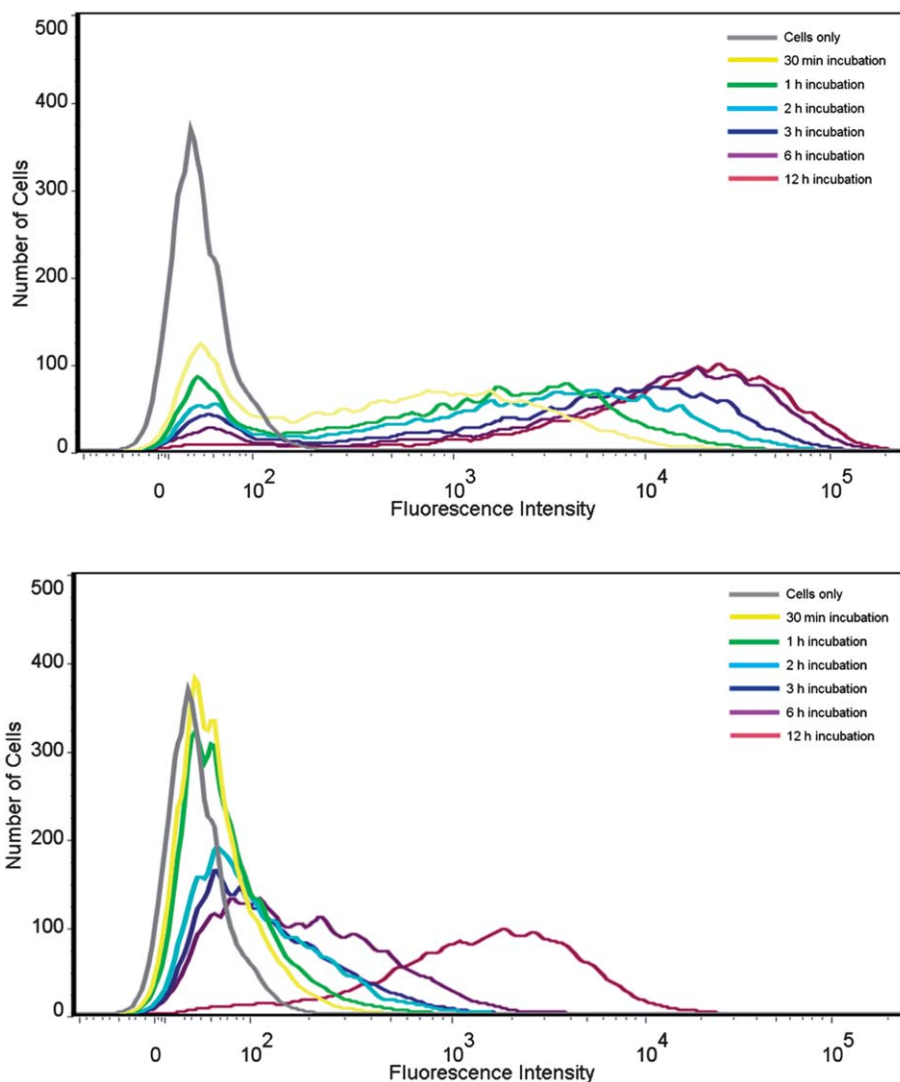
	KGG	KG
EtBr displacement (+/- charge ratio)	20 : 1	20 : 1
Diameter (nm)	348	424
Zeta potential (mV)	16.0	9.6
Repeat period (nm)	5.6	6.1

The aforementioned physicochemical studies described lipoplexes formed with DNA. Therefore, to further understand the differences in transfection outcomes, we evaluated siRNA lipoplexes using the **KGG** and **KG** lipopeptides as demonstrative examples. The experimental methods used were the same as those described earlier, and the results are summarized in Table 2.

The EtBr-siRNA fluorescence quenching assay showed that the maximum degree of EtBr displacement occurs at a charge ratio of about 20 : 1 for both **KGG** and **KG**, indicating that they have similar siRNA binding affinity. Compared to the lower

charge ratio needed to achieve maximum EtBr displacement in DNA binding assay, the ability to displace EtBr from siRNA is much weaker on a per lipid basis. The reason could be that the binding affinity to siRNA is weaker; or that the structural conformation of lipid bound to siRNA does not induce EtBr displacement compared to that of lipid-DNA structures at the same charge ratio.

The diameters of the lipoplex formed with siRNA were measured by DLS and were found to be 348 nm and 424 nm for **KGG** and **KG**, respectively, which were slightly larger than those formed with DNA. The associated zeta potentials (16.0 mV and 9.6 mV, respectively) were considerably lower than those of the DNA lipoplexes (35.6 mV and 40.8 mV). This suggests that siRNA may not be efficiently encapsulated into the supramolecular structure, resulting in negatively charged nucleic acid molecules being exposed at the surface. These lower zeta potentials could also be a contributing factor in increased aggregation and, therefore, larger lipoplex size. XRD patterns of **KGG** and **KG** in the presence of siRNA gave repeat periods of 5.6 nm and 6.1 nm, respectively, representing only relatively

**Fig. 7** Cellular uptake of rho-labeled DNA in CHO cells using (top) **KGG** and (bottom) **KG** with different incubation times at lipopeptide-DNA molar ratios of 12 : 1.

small differences compared to bilayers without nucleic acid present. Thus, as with DNA, siRNA is likely entrapped at the interface between liposomes.

The chemical and structural characterization described above shows that though there are measurable differences between lipoplexes formed from lipopeptide–DNA interactions and lipoplexes formed from lipopeptide–siRNA interactions, there is not a clear difference in structures formed with tripeptides compared to those with dipeptides (*i.e.*, **KGG** vs. **KG**). This is in contrast to our observations *in vitro*, which suggest that tripeptide-based liposomes are better vectors for DNA transfection compared to dipeptide liposomes, while both groups have shown variable success in siRNA knockdown. Consequently, we next determined if there was a difference in cellular uptake with the **KGG** and **KG** lipopeptides when complexed with DNA or siRNA. As before, these lipopeptides were chosen due to the similarities in physicochemical characteristics of their resulting liposomes and lipoplexes (similar T_m , X-ray diffraction pattern, size, and zeta potential as described

above), and the dramatic variations observed in transfection and knockdown experiments.

The cellular uptake of lipoplexes formed from **KGG** and **KG** lipopeptides with rhodamine-labeled DNA (rho-DNA) or rhodamine-labeled siRNA (rho-siRNA) into CHO cells were analyzed using flow cytometry to determine whether entry into the cell varied between these tri- and di-peptide systems (experimental details can be found in the ESI†). The **KGG** and **KG** lipids were tested at a molar ratio of 12 : 1 lipopeptide–DNA for DNA uptake experiments and a molar ratio of 15 : 1 lipopeptide–siRNA when measuring siRNA uptake. These ratios showed high transfection and knockdown efficiencies, respectively, and facilitated comparison of the DNA and siRNA uptake results as they use similar concentrations of lipopeptide applied to cells during incubation. As shown in Fig. 7, after 3 h of incubation with **KGG** lipoplexes (12 : 1 lipopeptide–DNA molar ratio) 75.8% of cells contain rho-DNA (as shown by the fluorescent signal above the baseline signal for untreated cells). On the other hand, **KG** lipoplexes (12 : 1 molar ratio) showed no

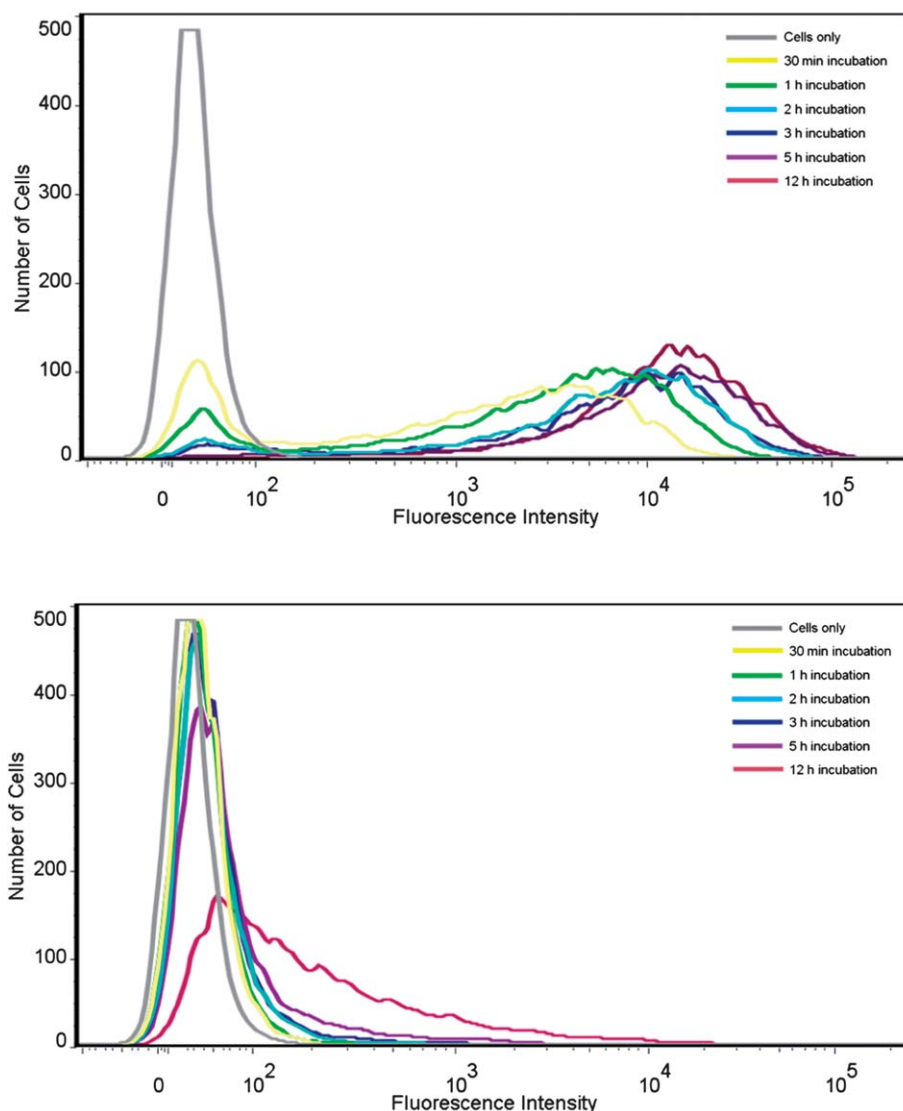


Fig. 8 Cellular uptake of rho-labeled siRNA in CHO cells using (top) **KGG** and (bottom) **KG** with different incubation times at lipopeptide–siRNA molar ratios of 15 : 1.

significant (1.2%) delivery of rho-DNA within 3 h. Total uptake after a 12 h incubation period was 91.8% and 66.6% for **KGG** and **KG**, respectively, compared to the positive control, Lipofectamine 2000 (90.2%; see ESI† for results of positive controls). Similar trends were observed when rho-siRNA was delivered *via* lipopeptide-based liposomes at an optimal knockdown lipopeptide-siRNA molar ratio of 15 : 1 (86.2% uptake with **KGG** and 0.5% uptake with **KG** after a 3 h incubation period; Fig. 8). After 12 h of incubation with siRNA-lipoplexes, the total uptake was 96.1% when using **KGG** and 11.5% when using **KG** (compared to 79.1% for Lipofectamine 2000).

These experiments show that there are differences between the cellular uptake kinetics of **KGG** and **KG** lipoplex systems, as well as differences between DNA- and siRNA-based lipoplexes. **KGG** lipoplexes were taken up by cells within 30 min for both DNA and siRNA systems, compared to the much slower delivery by **KG** (>6 h incubation necessary for substantial uptake to be observed). This trend suggests that the differences in transfection efficiency between lysine-based tripeptide- and dipeptide-DNA lipoplexes (Fig. 4) are due to the increased rate of cellular uptake when using the tripeptide (**KGG**), which promotes more intracellularly available DNA for nuclear entry and transcription. Specifically, using the tripeptide **KGG** results in increased cellular uptake and subsequently improved transfection, whereas dipeptide **KG** has limited transfection efficiency due to its low levels of DNA delivery into cells. For the cellular uptake data in regards to siRNA-based knockdown studies (Fig. 6), two factors should be considered. First, in agreement with previous studies showing that other siRNA lipoplexes are structurally different than those formed with DNA,^{47,48} we have found small differences in diameter, surface charge, and EtBr binding between our DNA-lipopeptides and siRNA-lipopeptides. It may be that these supramolecular structural discrepancies coupled with the variation in headgroup (one less glycine) cause the dipeptide (**KG**) to be better at cytoplasmic release of siRNA and subsequent knockdown than the tripeptide (**KGG**), despite the low quantity of total lipoplex uptake. Second, it has been observed that low quantities of siRNA localized in the cytoplasm are capable of effective interference outcomes, while DNA requires relatively higher quantities and transfer to the nucleus for successful transfection.^{49,50} It is this later point which is key, as low or high quantities of siRNA delivered to the cytoplasm affords efficient knockdown.

4 Conclusions

A series of lipids have been studied possessing different cationic tri- or di-peptides as headgroups. Compared to commercial transfection agents, the best of these lipids achieves similar *in vitro* transfection efficiency with low cytotoxicity. A number of observations can be made for these lipopeptides: (1) the physicochemical properties of the tri- and di-peptide lipoplexes formed with DNA are similar as they can bind to DNA and form bilayered vesicles with similar binding affinity, hydrodynamic size, zeta potential, transition temperature, and repeat period. (2) The **GGG** and **GG** lipopeptides, which possess relatively small headgroups and only 1 positive charge per molecule, form

liposomes with different sizes and transition temperatures than lipopeptides containing tri- or di-peptides with larger headgroups and 2 positive charges per molecule. (3) Lipopeptides with glycine-glycine spacing (**KGG**, **OGG**, and **RGG**) show good DNA transfection activity, while lipopeptides with a single glycine spacer (**KG**, **OG**, **RG**, and **GG**) exhibit only minimal DNA transfection capability *in vitro*. (4) In contrast, in siRNA transfection using either the tri- and di-lipopeptides afforded gene knockdown. (5) **KGG** and **KG** lipoplexes with siRNA form liposomes with slightly larger diameters and smaller zeta potentials than those formed with DNA. (6) The tri- and di-lipopeptides (**KGG** and **KG**) show very different cellular uptake kinetics for nucleic acid delivery *in vitro*, with the **KGG** lipoplexes being readily taken-up in cells compared to the **KG** lipoplexes. (7) Even a relatively small amount of siRNA delivered to cells can lead to significant transfection activity unlike with DNA, and thus both the tri- and di-lipopeptides are active for siRNA transfection.

In summary, this manuscript reports the synthesis and characterization of eight new lipopeptides with varying DNA and siRNA transfection efficiencies. The studies highlight the sensitivity of transfection efficiency to subtle changes in lipid chemical structure (*i.e.* spacing group and headgroup charge) and lipoplex supramolecular structure. Moreover, the lipoplex which performs the best for DNA transfection does not necessarily perform equally well for siRNA transfection. DNA transfection is more sensitive to the amount of lipoplex taken into cells compared to siRNA for resulting transfection. These peptide lipids add to the current body of synthetic transfection vectors, and along with results from other groups will provide insight into identification of an optimized vector for *in vitro* and *in vivo* delivery of DNA and siRNA.

Acknowledgements

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