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Biophysical Properties of CDAN/DOPE-Analogue Lipoplexes Account for Enhanced Gene Delivery

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Typically, cationic liposomes are formulated from the combination of a synthetic cationic lipid (cytofectin) and a neutral, biologically available co-lipid. However, the use of cationic liposome formulations to mediate gene delivery to cells is hampered by a paradox. Cationic lipids, such as N^1*-cholesteryloxycarbonyl-3-7-diazanonane-1,9-diamine (CDAN), are needed to ensure the formation of cationic liposome-DNA (lipoplex, LD) particles by plasmid DNA (pDNA) condensation, as well as for efficient cell binding of LD particles and intracellular trafficking of pDNA post-intracellular delivery by endocytosis. However, the same cationic lipids can exhibit toxicity, and also promote LD particle colloidal instability, leading to aggregation. This results from electrostatic interactions with anionic agents in biological fluids, particularly in vivo. One of the most commonly used neutral, bioavailable co-lipids, dioleoyl* L-α*-phosphatidylethanolamine (DOPE), has been incorporated into many cationic liposome formulations owing to its fusogenic characteristics that are associated with a preference for the inverted hexagonal* (H_{II}) *phase—a phase typical of membrane–membrane fusion events. However, these same fusogenic characteristics also destabilize LD particles substantially with respect to aggregation, in vitro and especially in vivo. Therefore, there is a real need to engineer more stable cationic liposome systems with lower cellular toxicity. We hypothesize that one way to achieve this goal should be to find the means to reduce the mol fraction of cationic lipid in cationic liposomes without impairing the overall transfection efficiency, by replacing DOPE with an alternative co-lipid with fusogenic properties "tuned" with a greater preference for the more stable lamellar phases than DOPE is able to achieve. Herein, we document the syntheses of triple bond variants of DOPE, and their formulation into a range of low charge, low cationic lipid containing LD systems. The first indications are that our hypothesis is correct in vitro.*

Introduction

Gene therapy may be described as the use of genes as medicines to treat disease, or more precisely as the delivery of nucleic acids by means of a vector (gene delivery vehicle) to patients for a therapeutic purpose. The putative mechanism of any given gene therapy depends upon the disease concerned and the nature of the nucleic acid delivered.[1–3] The vector may either be viral or nonviral (synthetic or physical) systems. Nowadays, progress is being made in all these areas of vector technology owing to improved biophysical characterizations of vector particles, and other insights from the mechanisms of disease and routes of delivery.[4] Cationic liposome-mediated nucleic acid delivery is a prevalent synthetic nonviral vector technology under development.[5,6] In comparison with delivery mediated by many other viral and physical nonviral methods, cationic liposome-mediated delivery appears to involve lower levels of immunogenicity, toxicity, and oncogenicity, not to mention potentially wider ranges of applications and more facile production. However, cationic liposome systems still lack efficacy in vivo. Nevertheless, there is a consensus that should cationic liposome vector systems be adequately matured with improved in vivo efficacy, then they may become the vector system of choice for many future gene therapy applications.

Typically, cationic liposomes are formulated from the combination of a synthetic cationic lipid (cytofectin) and a neutral, biologically available co-lipid (or "helper lipid"). A detailed understanding of the processes involved in cationic liposome-mediated plasmid DNA (pDNA) delivery and the clarification of specific physical and biochemical barriers encountered along the delivery pathway is necessary for the rational design of optimal synthetic cationic liposome vectors. These range from the biophysical aspects underlying the design and self-assembly properties of cationic liposome–pDNA complex (lipoplex; LD) particles to the different biological impediments that need to be overcome before gene expression can occur.[7–11] Cationic lipids are needed to ensure the formation of LD particles by pDNA condensation, as well as for efficient cell binding of LD particles and intracellular trafficking of pDNA post-intracellular delivery by endocytosis.[12] Furthermore, one of the most commonly used neutral, bioavailable co-lipids in cationic liposomes, dioleoyl L-α-phosphatidylethanolamine (DOPE), has been incorporated because of its associated fusogenic properties that may contribute towards efficient endosome-breakout (endosomolysis) and intracellular trafficking of pDNA.[6,13–20] These fusogenic effects are associated with a preference for

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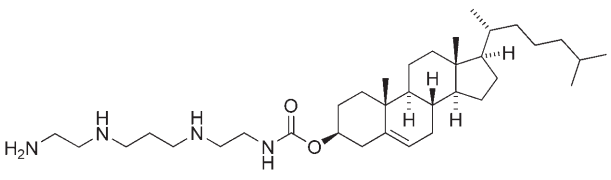
the inverted hexagonal (H_{II}) phase—a phase typical of membrane–membrane fusion events.

It is important to note here that the choice of co-lipid is not arbitrary in cationic liposome formulations. Replacement of the H_{II} phase lipid DOPE with the lamellar phase lipid dioleoyl L-α-phosphatidylcholine (DOPC) in otherwise identical cationic liposome formulations typically leads to a substantial reduction in transfection efficiency of the resultant lipoplexes.[17] Indeed, we have observed this result in our laboratories.[21] Unfortunately, cationic lipids are not only functionally useful but they are also known to exhibit toxicity and promote LD particle aggregation because of colloidal instability. This aggregation is a direct result from electrostatic interactions with anionic agents in biological fluids, particularly in vivo.[7] Likewise, the fusogenic characteristics of DOPE can also contribute significantly to LD particle instability with respect to aggregation in biological fluids and subsequent loss of cell delivery function, again especially in vivo.[22–24]

Given all these potential problems, we decided to attempt to engineer more stable LD particles using synthetic DOPE analogues. We reasoned that replacement of the kinked *cis*-Δ9 double bonds in the DOPE fatty acid tails with linear, triple bond functionalities would lead to improved intermolecular packing and an associated increase in lipid supramolecular stability, through increased lamellar-to-inverted-hexagonal (L_α/H_{II}) phase transition temperatures. Indeed, previous work has shown that triple bond-containing dialkynoyl lipids have a greater preference for the more stable lamellar phases, relative to the parent dioleoyl lipids.[25,26] Hence, our hypothesis was that replacement of DOPE in cationic liposome formulations with triple bond DOPE analogues could allow for the formulation of LD systems of greater stability and comparable transfection efficacy but lower toxicity due to an overall reduction in cationic charge made possible by a substantial reduction in the mol fraction (≪50 mol%) of cationic lipid present in formulations. Accordingly, we describe the synthesis of three novel triple-bond containing dialkynoyl DOPE analogue lipids, in which each *cis* double bond in the oleoyl fatty acid side chains are substituted by triple bonds in one of three locations. We then report upon the formulation of cationic liposomes and LD particles prepared from our new dialkynoyl DOPE analogue lipids and the well-characterized, well-known cationic lipid N^1-cholesteryloxycarbonyl-3-7-diazanonane-1,9-diamine (CDAN).[27–31] The observed changes in biophysical and



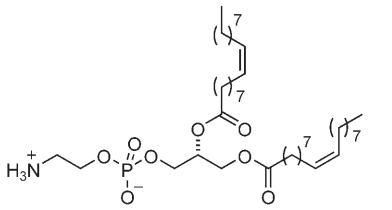
CDAN

transfection properties of such systems relative to those of CDAN/DOPE (1:1, *m*/*m*) parent cationic liposomes and LD particles suggest that our hypothesis may be correct in vitro.

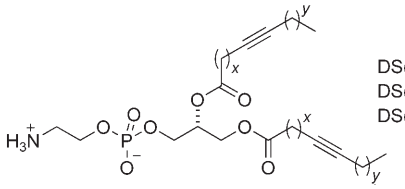
Results and Discussion

Chemistry

Our proposed modifications to DOPE (**1**) are shown, replacing the *cis* double bonds at position 9 of the fatty acid alkyl chains with triple bonds at positions 4, 9, and 14, giving compounds

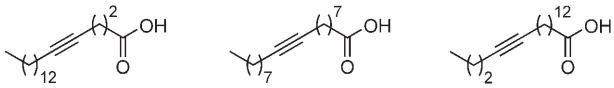


DOPE (**1**)



DS(4-yne)PE (**2**; *x* = 2, *y* = 12)
DS(9-yne)PE (**3**; *x* = 7, *y* = 7)
DS(14-yne)PE (**4**; *x* = 12, *y* = 2)

2, **3**, and **4**, respectively. The syntheses of these DOPE analogues (**2**–**4**) were achieved in a two-step procedure from the respective alkynoic acids, whose structures are shown and



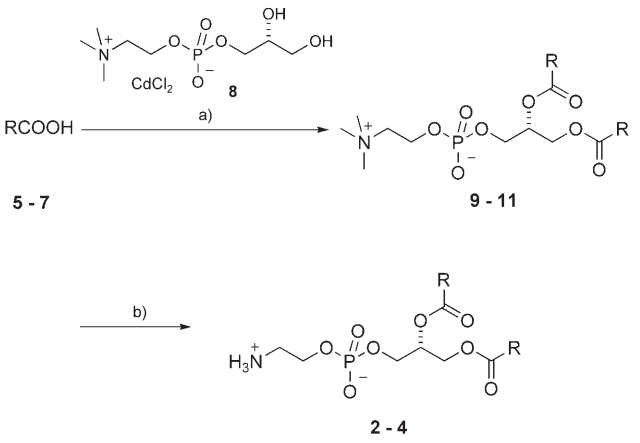
5 **6** **7**

whose syntheses have been reported previously.[25,26] Activation of fatty acid **5**, **6**, or **7** with carbonyl di-imidazole (CDI) enabled smooth esterifications of L-α-glycerophosphocholine. $CdCl_2$ (**8**) to take place in all cases (Scheme 1), thereby furnishing the corresponding phosphatidylcholine (PC) molecules **9**–**11** in moderate to good yields (57–67%). Subsequent enzymatic transphosphatidylation[32] of PC lipids **9**, **10**, and **11** with phospholipase D (PLD) in the presence of ethanolamine afforded the desired phosphatidylethanolamine (PE) molecules distear-4-ynoyl L-α-phosphatidylethanolamine [DS(4-yne)PE] (**2**), distear-9-ynoyl L-α-phosphatidylethanolamine [DS(9-yne)PE] (**3**), and distear-14-ynoyl L-α-phosphatidylethanolamine [DS(14-yne)PE] (**4**), respectively. The yields and purities of these DOPE analogues are given in Table 1.

Biophysical characterization

Photon correlation spectroscopy (PCS)

Photon correlation spectroscopy (PCS) was used to obtain an average size distribution for liposomes containing CDAN and DOPE or CDAN and DOPE analogues at various molar ratios. Liposome diameters were not found to vary too substantially



Scheme 1. a) 1: carbonyl di-imidazole (CDI), $CHCl_3$, room temperature, 30 min; 2: **8**, DBU, DMSO, room temperature, 7 h; b) ethanolamine, phospholipase D (PLD), $CHCl_3$, 100 mM NaOAc/50 mM $CaCl_2$, pH 6.5, 30 °C, 3 h.

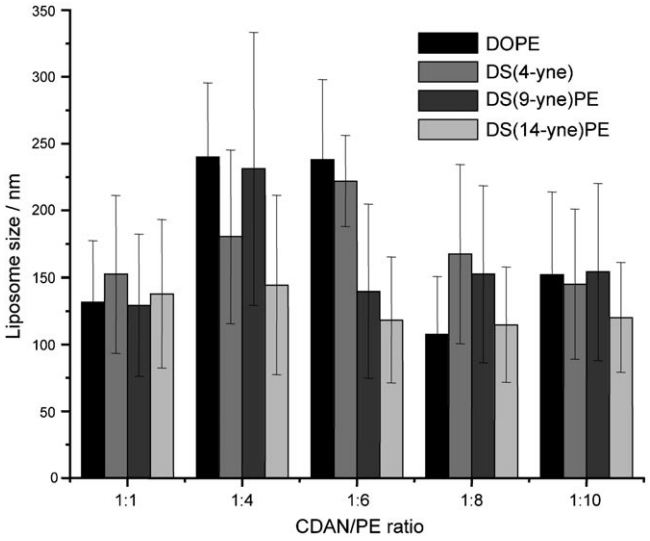


Figure 1. Size distribution for liposomes containing CDAN with DOPE or DOPE analogues at various molar ratios.

Table 1. Yields for the syntheses of the dialkynoyl DOPC analogues and for their conversions to the corresponding DOPE analogues.

Acid	DOPC analogue	Yield [%]	DOPE analogue	Yield [%]	Purity [%][a]
5	**9**	57	**2**	92	>98
6	**10**	67	**3**	94	>98
7	**11**	59	**4**	93	>98

[a] Purity was determined by HPLC (see Experimental Section).

in size with CDAN/PE ratio (Figure 1). This uniform distribution in size may be attributed to the efficacy of sonication to clarity at 50 °C that overcomes most of the size-dependent variation in liposomes as a function of lipid mol%. Standard LD particles were prepared (lipid/pDNA, 12:1, *w/w*) with diameters equivalent to their corresponding cationic liposome diameters (results not shown). Therefore, we are confident that LD particle size cannot play a significant role in varying transfection efficiency.

X-ray diffraction studies

Small angle and wide angle X-ray diffraction patterns were studied to probe liposome and LD particle self-assembly. Predominant structures that emerged are depicted in Figure 2. These are the inverted hexagonal (H_{II}) phase and two lamellar phase structures (L_α and $L_{\beta'}$), which have been identified previously through the analyses of diffraction peak positions.[33–35] Results from temperature-related structural studies involving liposomes prepared from either DOPE or one of the dialkynoyl DOPE analogues are shown in Figure 3. As reported previously, liposomes formulated from 100% DOPE always prefer to adopt the H_{II} phase.[36] Herein we were able to verify this finding, noting that liposomes comprising 100% DOPE formed the inverted hexagonal structure over the temperature range 5 to 95 °C, with a mean distance between adjacent lipid rods (d_{lipid}) of 31.6 Å according to Equation (1):

$$d_{lipid} = \frac{4\pi}{\sqrt{3}\, q_{001}} \quad (1)$$

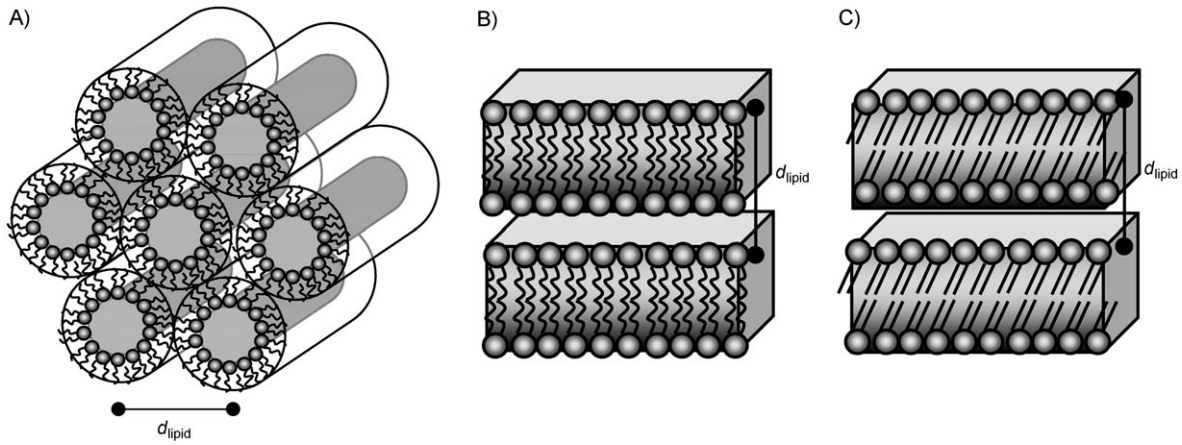


Figure 2. Self-assembled structures of liposomes: A) inverted hexagonal (H_{II}); B) fluid-chain lamellar (L_α); C) chain-ordered lamellar ($L_{\beta'}$).

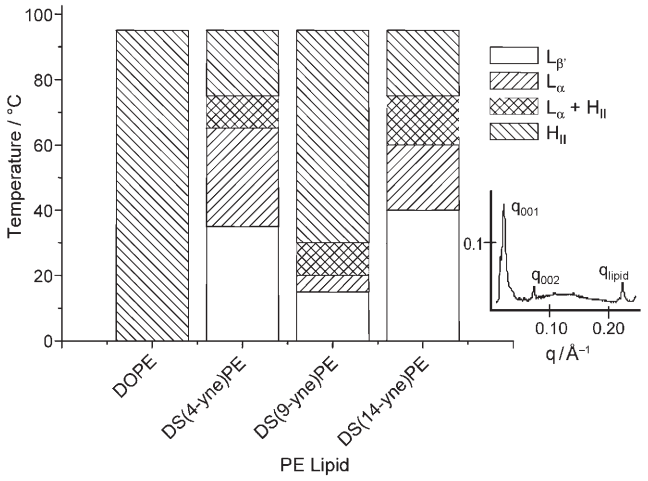


Figure 3. Thermotropic phase diagram for DOPE and DOPE analogues as determined by X-ray diffraction with liposomes at a concentration of 10 mg mL^{-1}. DOPE shows H_{II} phase for all temperatures whereas the analogues are in lamellar phases at lower temperatures. Inset: typical X-ray diffraction pattern from liposomes where the q_{001} and q_{002} result from the lamellar structure and q_{lipid} results from the lipid chain ordering.[35]

Turning to the dialkynoyl DOPE analogues, very similar behavior was observed with liposomes comprising 100% of either of the two analogues DS(4-yne)PE or DS(14-yne)PE, where the triple bonds are located high up on the fatty acid alkyl chains or lower down the chains, respectively (Figure 3). Both liposome systems appeared to favor lamellar structures, especially at lower temperatures, where the fatty chains were seen to be close-packed, forming the $L_{\beta'}$ structure with a d_{lipid} spacing between lipid bilayers of 30 Å. As the temperature was increased, the fatty acid alkyl chains were seen to enter the fluid phase, L_α, driven by the need to increase system entropy. Interestingly, subsequent entry into the H_{II} phase was considerably delayed and only glimpsed at $>60\,^{\circ}C$, where the distance d_{lipid} between adjacent lipid rods was 32.0 Å. Quite clearly, simple modifications in chemical structure can alter thermotropic phase properties rather drastically, leading to the modification of phase diagrams by the incorporation of at least two additional lamellar phases. The fact that the thermotropic phase behaviors of both DS(4-yne)PE or DS(14-yne)PE were approximately equivalent suggests that replacement of the *cis* double bond with a triple bond actually enables fatty acid alkyl chain packing to become more ordered irrespective of triple bond location.

By contrast, DS(9-yne)PE liposomes were found to exhibit more biologically relevant phase behavior. In this instance, the DOPE fatty acid *cis* double bonds were replaced with triple bonds at the equivalent chain position. Once again, the lamellar structure $L_{\beta'}$ was favored by 100% DS(9-yne)PE liposomes at low temperature, followed by a shift to the L_α phase with an increase in bilayer spacing d_{lipid} up to 30 Å, albeit at a much lower temperature than was observed with DS(4-yne)PE or DS(14-yne)PE liposomes. Thereafter, L_α and H_{II} phases were observed to co-exist quite readily near physiologically relevant temperatures, suggesting that the DS(9-yne)PE lipid could have an especially important impact on the biological activities of corresponding LD particles. Clearly, DS(9-yne)PE liposomes do not exhibit the same thermotropic behavior as DS(4-yne)PE or DS(14-yne)PE liposomes, but then neither do DS(9-yne)PE liposomes exhibit the same behavior as DOPE liposomes.

When studying the X-ray diffraction patterns for the corresponding LD particles formulated using cationic lipid CDAN at different CDAN/PE ratios (Figure 4), the influence of the cationic lipid on phase behavior could be seen, especially at high CDAN/PE ratios. In this instance, all systems were seen to adopt a lamellar structure at the physiological temperature, 37 °C, regardless of the PE lipid used, certainly owing to the influence of CDAN on LD particle structure. We observed that LD particles containing the DOPE analogues DS(4-yne)PE and DS(14-yne)PE remained in the lamellar phase even as the CDAN/PE ratio was decreased, with DNA double helices intercalated between the lipid bilayers.[34] In fact, there appeared to be coexistence between the L_α and $L_{\beta'}$ phases at all CDAN/PE ratios with lipid bilayer spacings d_{lipid} of 55 Å for the $L_{\beta'}$ phase, and d_{lipid} of 71 Å for the L_α phase. In contrast, where DOPE or DS(9-yne)PE were involved, then the inverted hexagonal (H_{II}) structure was adopted by LD particles at CDAN/PE ratios less than 1:4 (*m*/*m*) with DNA double helical rods now sited within the lipidic tubes.[33]

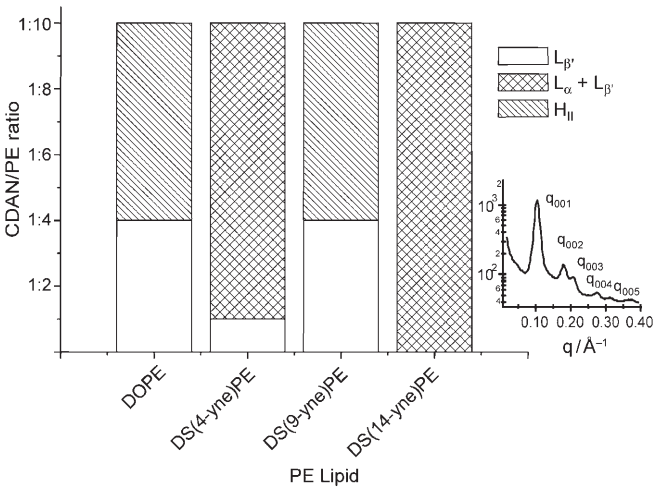


Figure 4. Phase diagrams for CDAN and DOPE or CDAN and DOPE analogue LD particles at various cationic lipid to neutral lipid ratios, as determined by X-ray diffraction at 37 °C. Inset: typical X-ray diffraction pattern from LD particles where all peaks result from the inverted hexagonal (H_{II}) structure. Liposomes were at 10 mg mL^{-1}, DNA at 5 mg mL^{-1}, and LDs were formulated at a lipid to DNA weight ratio of 12:1, with each sample containing 0.2 mg DNA.

However, even though the macroscopic behaviors of CDAN/DOPE and CDAN/DS(9-yne)PE LD particles appear similar (Figure 4), local microscopic behavior should be nonequivalent given differences in the X-ray diffraction profiles of the corresponding PE liposomes alone (Figure 3). In other words, CDAN/DS(9-yne)PE LD particles appear to have similar macroscopic, average thermotropic behavior to CDAN/DOPE LD particles, but DS(9-yne)PE alone has a much higher propensity to adopt the more stable lamellar phase than DOPE. Therefore, we pro-

pose that, hypothetically, CDAN/DS(9-yne)PE LD particles may benefit from the existence of transient lamellar phase "patches" (or "rafts") in DS(9-yne)PE-rich regions at physiologically relevant temperatures that may confer some LD particle stability with respect to aggregation and potential loss of transfection efficacy.

Biological activity

In vitro studies

To ascertain the functional efficiency of the LD systems containing DOPE analogues, in vitro transfection experiments were performed on the cancer cell line Panc-1, originating from human pancreatic cells. For details on procedures, please see the experimental methods section. Briefly, Panc-1 cells were cultured in 24-well plates until they reached approximately 70–80% confluency. As the main goal of this research was to formulate lower-charged (less mol% CDAN) cationic liposomes, and hence lower-charged lipoplexes, with minimal negative impact on transfection efficiencies through the addition of DOPE analogues with modulated fusogenicities, LD particles were formulated at a variety of molar ratios of cationic lipid to neutral lipid with pDNA (harboring the luciferase gene), at a lipid to DNA ratio of 12:1 (*w*/*w*). Transfection was effected in serum free cell media for 5 h, after which time cells were washed rigorously with phosphate buffered saline (PBS) solution, and further incubated for 24 h to allow for luciferase gene expression to take place. Transfection efficiency was judged to be proportional to the level of enzyme activity measured in cells post 24 h. Enzyme activity levels were quantified on a Berthold luminometer and normalized with respect to total remaining cellular proteins by a standard protein BCA assay.

It is important to address here several biophysical parameters before discussing the transfection data. First, zeta potential data confirmed that cationic liposomes were significantly positively charged at both ends of the formulation series. As a representative example of the zwitterionic and isomeric PE lipids, the surface charges of CDAN/DS(9-yne)PE liposomes were studied and were found to fall from $+93.4 \pm 3.4$ mV for 1:1 (*m*/*m*) formulations to $+62.4 \pm 0.7$ mV for 1:10 (*m*/*m*) formulations, consistent with reduced cationic lipid content. Additionally, we have previously calculated N:P ratios (number of protonatable amines : number of phosphates), which are significantly >1 for all the LD complexes studied, suggesting that all LD particles used for transfection experiments were positively charged.[26] Furthermore, as the calculated N:P ratios are significantly positive, then complexation of DNA should be total, resulting in complete gel retardation of DNA mobility, as has been reported by us previously.[28] Hence, DNA dose per transfection experiment can be expected to be uniform within experimental error. Accordingly, there is unlikely to be an effective variation in the dose of DNA delivered to cells between transfection experiments that could otherwise have an obvious impact on transfection efficiency.

Transfection data (Figure 5) clearly illustrate the dominance of DS(9-yne)PE-containing LD systems. By contrast, the behavior of LD particles containing DOPE or one of the other two DOPE analogues DS(4-yne)PE and DS(14-yne)PE was less effec-

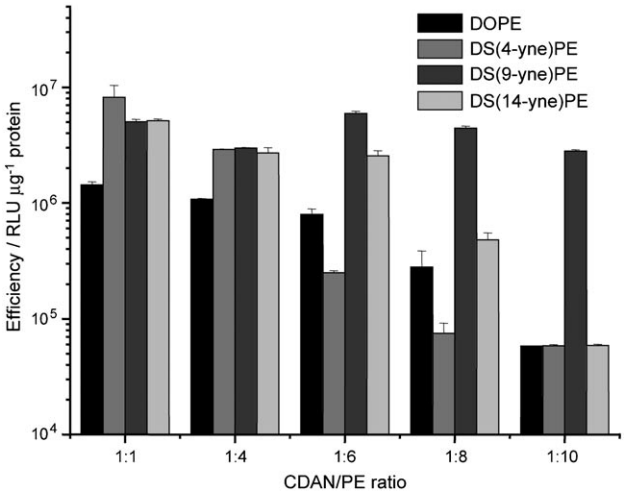


Figure 5. In vitro transfection efficiency. Liposomes were at 0.5 mg mL^{-1}, DNA at 1 mg mL^{-1}, and LD particles were formulated at a lipid to DNA weight ratio of 12:1, with each sample containing 0.5 µg DNA. Control data: untreated cells = 0.423165 ± 0.099548 RLU per µg protein; naked DNA = 41.60995 ± 5.249062 RLU per µg protein.

tive. Transfection efficiency was found to decline with increasing mol% of DOPE or DOPE analogue. This trend can be accounted for by the declining influence of the cationic lipid on transfection as mol% incorporation was reduced, which makes sense given the well-known importance of the cationic lipid and electrostatic interactions in effecting efficient transfection.[37] Surprisingly, the efficacy of transfection mediated by LD particles containing DS(9-yne)PE remained broadly constant with increasing mol% of DS(9-yne)PE at the expense of mol% of CDAN cationic lipid. Arguably, the unusual thermotropic phase transition behavior exhibited by 100% DS(9-yne)PE liposomes at physiologically relevant temperatures, in comparison to the corresponding behavior in 100% DOPE and other PE liposomes (Figure 3), might, in part, account for this observation. Specifically, the data (Figure 5) suggest that the reduced cationic lipid content was cleanly compensated for by the inclusion of a more lamellar phase stable, yet still H_{II} phase accessible, DOPE analogue, DS(9-yne)PE, thereby ensuring that transfection efficiency was retained.

Subsequently, LD transfections were observed and compared in the presence of 10% serum (Figure 6), an environment that begins to emulate in vivo conditions. The procedure for such experiments was identical to previous transfection experiments, although this time transfections were conducted instead in the presence of 10% fetal calf serum (FCS). In this case, LD particles prepared from DOPE analogues were found to exhibit enhanced transfection efficiencies over those prepared from DOPE, in all cases where the CDAN/PE molar ratio was below 1:1 (*m*/*m*). Overall, CDAN/DOPE-analogue LD trans-

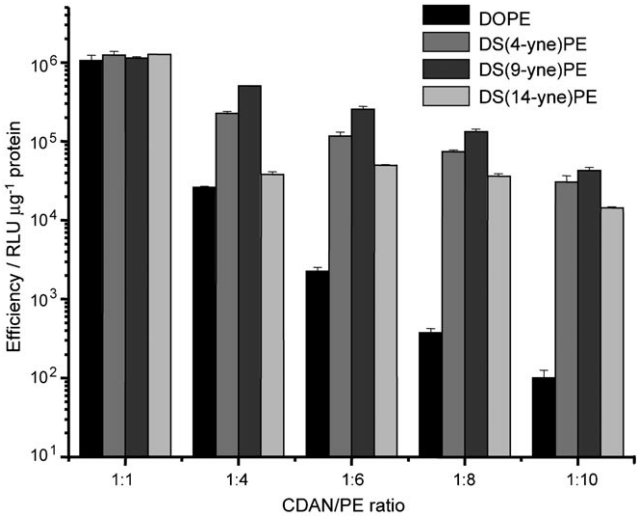


Figure 6. In vitro transfection efficiency in the presence of 10% fetal calf serum (FCS). Liposomes were at 0.5 mg mL^{-1}, DNA at 1 mg mL^{-1}, and LDs were formulated at a lipid to DNA weight ratio of 12:1, with each sample containing 0.5 μg DNA. Control data: untreated cells = 0.423165 ± 0.099548 RLU per μg protein; naked DNA = 41.60995 ± 5.249062 RLU per μg protein.

fections in the presence of serum (Figure 6) were found to be modestly less efficient in comparison with equivalent LD transfections performed in the absence of serum (Figure 5). However, CDAN/DOPE LD transfections in the presence of serum were substantially reduced by four orders of magnitude compared with equivalent LD transfections in the absence of serum (Figure 5). Such clear differences in behavior can be related directly to the individual thermotropic phase behaviors of LD particles (Figure 4). CDAN/DS(4-yne)PE and CDAN/DS(14-yne)PE LD particles were shown to be uniformly lamellar in behavior over all CDAN/PE ratios. Therefore, we would suggest that these biophysical properties were endowing sufficient additional LD particle stability so as to overcome the negative effects of biological fluids (serum) on LD particle stability and transfection efficacy.

By contrast, Figure 4 shows that CDAN/DOPE LD particles adopted the inverted hexagonal (H_{II}) phase from CDAN/PE ratios of 1:4 (*m*/*m*) and lower, therefore these particles would be expected to be much more susceptible to instability in the presence of serum, consistent with the substantial decline in transfection efficiency observed in Figure 6. However, CDAN/DS(9-yne)PE LD particles also adopted the H_{II} phase from CDAN/PE ratios of 1:4 (*m*/*m*) and yet they behaved as other CDAN/DOPE-analogue LD systems, rather than as CDAN/DOPE LD systems. A possible, hypothetical explanation for this substantial difference in transfection behavior may be the existence of transient lamellar "patches" at physiologically relevant temperatures in DS(9-yne)PE-rich regions of CDAN/DS(9-yne)PE LD particles (in isolation, DS(9-yne)PE adopted both the fusogenic H_{II} phase and the stable L_α phase, as indicated in Figure 3). These could act to partially stabilize CDAN/DS(9-yne)PE LD particles in the presence of serum with respect to aggregation and colloidal instability. In contrast, CDAN/DOPE LD particles should not have access to any such benefits (in isolation, DOPE adopted the fusogenic H_{II} phase at all temperatures, Figure 3). Overall, these serum transfection data illustrate clearly the benefits of all DOPE analogues to LD transfection, as stable, transfection competent LD systems can now be prepared with limited cationic lipid (CDAN) content by mol%. These transfection data also appear to underscore our previous data and conclusions concerning the central importance of lipids that favor lamellar phase structure to stabilize LD particles and retain transfection or even enhance transfection efficiency.[25,26]

Cell viability studies

One of the main drawbacks of using cationic liposome-mediated delivery can be cellular toxicity introduced with the charge of the cationic lipid;[38] it is therefore of interest to reduce the amount of cationic lipid in LD formulations without impairing transfection. Our data in Figures 5 and 6 suggest we have achieved this goal through the novel DOPE analogues **2**–**4**. Thus, we next investigated the cell viability post-transfection to examine how toxic LD formulations were to Panc-1 cells. Results from these studies are shown (Figure 7), where the percentage of cell viability was ascertained through comparison with untreated cells. Experimental procedures were identical to the serum-free in vitro transfections, although instead of protein quantification, an MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay was performed to assess cell viability. As expected, toxicity was found to drop and cell viability increased as the amount of cationic lipid (charge) was reduced in the LD formulations. Importantly, the toxicity data also show that at low CDAN/PE molar ratios, the triple bonds in the PE analogues had little impact on cellular toxicity, relative to DOPE, but, as shown in Figure 5 and 6, these lipids had substantially beneficial effects on transfection.

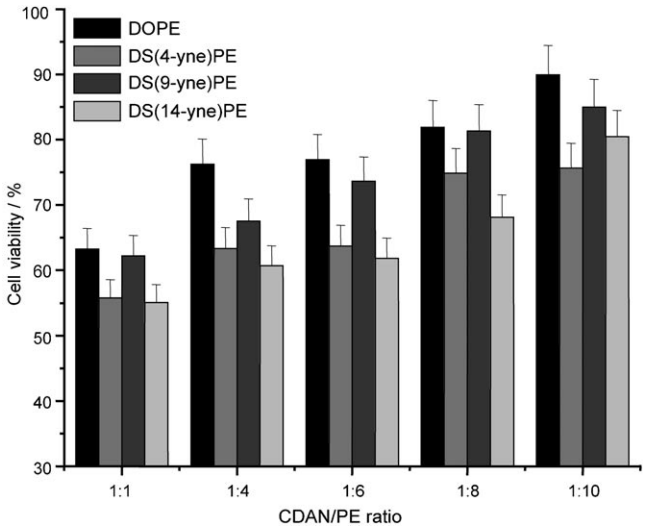


Figure 7. In vitro cell viability assay (MTT). Liposomes were at 0.5 mg mL^{-1}, DNA at 1 mg mL^{-1}, and LDs were formulated at a lipid to DNA weight ratio of 12:1, with each sample containing 0.5 μg DNA.

Conclusions

We have shown that the replacement of the DOPE fatty acid *cis*-Δ9 double bonds with triple bonds can lead to wide variations in the biophysical properties of their liposomes and their LD particles. In particular, we have observed variations in the phase behavior, with transitions between lamellar (L_α) and inverted hexagonal (H_{II}) phases occurring at different temperatures depending upon the molecular structures of the lipids used. We have then demonstrated that these differences in biophysical properties may be put to use for the formulation of lower-charged LD particles that, despite being formulated with only low mol% values of cationic lipid, retain transfection efficacy for Panc-1 cells. We propose that where these newly formulated LD particles retain transfection efficiency, they are benefiting from an observed, increased tendency, relative to DOPE, for constituent dialkynoyl DOPE analogue co-lipids to adopt lamellar-phase behavior within complex particles in preference to the less stable, fusogenic H_{II} phase, thereby gaining increased stability with respect to aggregation.

In serum-free conditions, the DS(9-yne)PE analogue emerged as the preferred neutral co-lipid to assist in vitro transfection of Panc-1 cells involving LD particles formulated with low mol% values of cationic lipid. In the presence of serum, all three analogues, DS(4-yne)PE, DS(9-yne)PE, and DS(14-yne)PE, appeared able to assist in vitro transfection of Panc-1 cells. Moreover, these low cationic charge LD particles also appeared to be somewhat less toxic towards Panc-1 cells, consistent with the lower mol% of cationic lipid. Data from these studies are important as they suggest that triple bond analogues of the neutral co-lipid DOPE could be useful chemical components to engineer the generation of stable, transfection-competent LD particles with minimal cationic lipid compositions that simultaneously confer the likelihood of lower cellular toxicities. Our current in vitro data set now needs to be extended to in vivo studies. Overall, we appear to have developed a new series of LD systems that could be useful for certain in vitro applications and may also be directly applicable for certain applications in vivo as well.[38]

Experimental Section

General: N^1-Cholesteryloxycarbonyl-3–7-diazanonane-1,9-diamine (CDAN) and dioleoyl-L-α-phosphatidylethanolamine (DOPE) were obtained from Avanti Polar Lipids (Alabaster, AL, USA). CH_2Cl_2 was distilled over P_2O_5, other solvents were purchased predried as required. Reactions were performed in oven-dried glassware under an atmosphere of argon. Thin layer chromatography (TLC) was performed on precoated Merck-Kieselgel 60 F_{254} aluminum backed plates and revealed with acidic ammonium molybdate (IV), basic potassium manganate(VII), or other agents as appropriate. Flash column chromatography was accomplished on Merck-Kieselgel 60 (230–400 mesh). All other chemicals were of analytical grade or the best grade available and were purchased from Sigma–Aldrich (UK) and Lancaster. Solvent mixtures used for column chromatography of lipids: solvent A (CH_2Cl_2:MeOH:H_2O, 77.54:23.23:2.23) and solvent B (CH_2Cl_2:MeOH:H_2O, 65:25:4). ^1H and ^{13}C NMR spectra were recorded either by using a Bruker DRX300, Joel GX-270Q, or Bruker Avance 400 with residual isotopic solvent ($CDCl_3$, $\delta_H = 7.27$ ppm, $\delta_C = 77.0$ ppm) as internal reference. Mass spectra were obtained by using VG-070B, Joel SX-102, or Bruker Esquire 3000 ESI instruments. IR spectra were obtained on a JASCO FT/IR-620 infrared spectrometer. UV spectroscopy was conducted by using a Pharmacia Biotech Ultrospec 4000 spectrometer at defined wavelengths. Analytical HPLC (Hitachi-LaChrom L-7150 pump system equipped with a Polymer Laboratories PL-ELS 1000 evaporative light scattering detector) was conducted with a HICHROM KR60-5-2493 silica column, with mobile phase A ($CHCl_3$:MeOH:NH_4OH, 80:19.5:0.5) and mobile phase B ($CHCl_3$:MeOH:H_2O:NH_4OH, 60:34:5.5:0.5), A/B, 0–6 min [100/0], gradient 6–28 min [0/100], 28–38 min [0/100], gradient 38–40 min [100/0], 40–60 min [100/0].

General procedure for preparation of phosphatidylcholine (PC) lipids (9, 10, or 11): carbonyl di-imidazole (CDI; 3.33 equiv) was added to a stirred solution of the fatty acid (**5**, **6** or **7**; 2.67 equiv) in anhydrous $CHCl_3$ (0.24 M). This solution was allowed to stir for 30 min, during which time L-α-glycerophosphocholine$CdCl_2$ (**8**; 1 equiv) was dissolved in anhydrous DMSO (0.09 M), with a little heating (40 °C). DBU (2.67 equiv) was added dropwise to the solution of the phosphocholine, then the $CHCl_3$ solution of the acid imidazolide was transferred to the DMSO solution via a cannula. Periodically, the reaction mixture was warmed in a water bath to approximately 40 °C for about 30 min, then the reaction was stirred for 7 h at room temperature. The reaction mixture was neutralized with 0.1 M acetic acid, then extracted into a 2:1 mixture of $CHCl_3$:MeOH, and washed with a 1:1 mixture of H_2O:MeOH (×5), back-extracting each time. The subsequent organic fractions were combined, and concentrated in vacuo, azeotroping the water with methanol as required. The residual orange–brown, viscous oil was purified by silica gel flash column chromatography (solvent A), to give the PC molecules as off-white, waxy solids.

Distear-4-ynoyl L-α-phosphatidylcholine (DS(4-yne)PC, 9): (335 mg, 57%); R_f 0.45 [solvent B]; ^1H NMR (270 MHz; $CDCl_3$): $\delta =$ 0.86 (t, $J = 6.5$ Hz, 6H; $2CH_2CH_3$), 1.15–1.49 (2m, 44H; $22CH_2$), 2.05–2.14 (m, 4H; $2CH_2C{\equiv}CCH_2CH_2CO_2$), 2.36–2.57 (m, 8H; $2CH_2CH_2CO_2$), 3.33 (s, 9H; $N(CH_3)_3$) 3.77 (m, 2H; $CH_2N(CH_3)_3$), 3.92 (m, 2H; glycerol-C3-$H_{a,b}$), 4.14 (dd, $J = 11.9$, 7.2 Hz, 1H; glycerol-C1-H_b), 4.34–4.42 (m, 2H; $CH_2CH_2N(CH_3)_3$), 4.38 (1H; dd, $J = 12.1$, 2.7 Hz, glycerol-C1-H_a), 5.20 (1H; m, glycerol-C2-H); ^{13}C NMR (100 MHz; $CDCl_3$) $\delta = 14.5$ (2 signals), 15.0, 15.1, 19.1, 23.1, 29.4, 29.4, 29.4, 29.5, 29.6, 29.8, 30.0, 30.1, 30.1, 32.3 (26 signals), 34.3, 34.4, 54.7, 59.7 (d, $J = 3.9$ Hz), 63.5, 63.7 (d, $J_{CP} = 4.7$ Hz), 66.6 (d, $J_{CP} = 6.5$ Hz), 71.2 (d, $J_{CP} = 7.0$ Hz), 78.2, 81.6, 81.6 (4 signals), 172.0, 172.3; ν_{max} (nujol mull) 2235, 1723, 1683, 1424, 1373, 1235 cm^{-1}; HRMS (FAB^+) calcd for $[C_{44}H_{80}NO_8P+H]^+$ 782.569983, found 782.572556; HPLC $t_R = 11.5$ min.

Distear-9-ynoyl L-α-phosphatidylcholine (DS(9-yne)PC, 10): (473 mg, 67%); R_f 0.47 [solvent B]; ν_{max} (nujol mull) cm^{-1} 2237, 1709, 1626, 1451, 1279, 1246; δ_H (270 MHz; $CDCl_3$) 0.81 (6H; t, $J =$ 6.8 Hz, $2CH_2CH_3$), 1.17–1.58 (44H; 2m, $22CH_2$), 2.04–2.11 (8H; m, $2CH_2C{\equiv}CCH_2$), 2.36–2.57 (4H; m, $2CH_2CO_2$), 3.33 (9H; s, $N(CH_3)_3$), 3.74 (m, 2H; $CH_2N(CH_3)_3$), 3.87 (m, 2H; glycerol-C3-$H_{a,b}$), 4.05 (1H; dd, $J = 12.0$, 7.2 Hz, glycerol-C1-H_b), 4.24 (m, 2H; $CH_2CH_2N(CH_3)_3$), 4.33 (1H; dd, $J = 12.0$, 2.4 Hz, glycerol-C1-H_a), 5.13 (1H; m, glycerol-C2-H); δ_C (100 MHz; $CDCl_3$) 14.5 (2 signals), 19.1, 23.0, 25.2, 25.3, 29.1, 29.3, 29.4, 29.4, 29.5, 29.6, 29.6, 32.2 (26 signals), 34.5, 34.7, 54.8, 59.7 (d, $J = 4.4$ Hz), 63.4, 63.7 (d, $J_{CP} = 4.9$ Hz), 66.7 (d, $J_{CP} =$ 5.9 Hz), 70.9 (d, $J_{CP} = 7.6$ Hz), 80.4, 80.7 (4 signals), 173.5, 173.9; HRMS (FAB^+) calcd for $[C_{44}H_{80}NO_8P+H]^+$ 782.569983, found 782.571487; HPLC $t_R = 11.5$ min.

Distear-14-ynoyl L-α-phosphatidylcholine (DS(14-yne)PC, 11): (420 mg, 59%); R_f 0.44 [solvent B]; ν_{max} (nujol mull) cm^{-1} 2269, 1701, 1672, 1497, 1322; δ_H (270 MHz; $CDCl_3$) 0.94 (6H; t, $J=7.4$ Hz, $2CH_2CH_3$), 1.17–1.62 (44H; 2 m, $22CH_2$), 2.05–2.18 (8H; m, $2CH_2C\equiv CCH_2$), 2.25–2.31 (4H; m, $2CH_2CO_2$), 3.33 (9H; s, $N(CH_3)_3$), 3.81 (m, 2H; $CH_2N(CH_3)_3$), 3.93 (m, 2H; glycerol-C3-$H_{a,b}$), 4.10 (1H; dd, $J=$ 11.9, 7.4 Hz, glycerol-C1-H_b), 4.30 (m, 2H; $CH_2CH_2N(CH_3)_3$), 4.37 (1H; dd, $J=12.1$, 2.7 Hz, glycerol-C1-H_a), 5.18 (1H; m, glycerol-C2-H); δ_C (100 MHz; $CDCl_3$) 13.9 (2 signals), 19.1, 21.1, 22.9, 25.3, 25.4, 29.3, 29.6, 29.7, 29.7, 29.8, 29.9, 30.0, 30.0 (26 signals), 34.5, 34.7, 54.7, 59.7 (d, $J=4.6$ Hz), 63.4, 63.7 (d, $J_{CP}=4.8$ Hz), 66.6 (d, $J_{CP}=6.5$ Hz), 70.9 (d, $J_{CP}=7.5$ Hz), 80.4, 80.7 (4 signals), 173.5, 173.9; HRMS (FAB^+) calcd for $[C_{44}H_{80}NO_8P+H]^+$ 782.569983, found 782.568970; HPLC $t_R=11.5$ min.

General procedure for preparation of phosphatidylethanolamine (PE) lipids (2, 3, or 4): A solution of ethanolamine (6 equiv) in a 100 mM NaOAc/50 mM $CaCl_2$ buffer (1.89 M ethanolamine) at pH 6.5 (pH adjusted with acetic acid), was added to a stirred solution of the PC lipid (**9**, **10**, or **11**; 1 equiv) in $CHCl_3$ (0.05 M PC lipid) at 30 °C. Phospholipase D (PLD; 578 units $mmol^{-1}$ of PC lipid, at a concentration of 250 units mL^{-1} of the aforementioned buffer, pH 6.5) was added to this biphasic system and the reaction mixture was stirred at 30 °C for 3 h. The crude organic material was extracted by washing with $CHCl_3$:MeOH, 2:1 (x 3). The organic layers were combined and washed with H_2O, then concentrated in vacuo, and subjected to silica gel flash column chromatography (solvent A) to purify, furnishing the PE lipids as off-white, waxy solids.

Distear-4-ynoyl L-α-phosphatidylethanolamine (DS(4-yne)PE, 2): (170 mg, 92%); R_f 0.56 [solvent B]; ν_{max} (nujol mull) cm^{-1} 2238, 1714, 1424, 1291, 1101; δ_H (270 MHz; $CDCl_3$) 0.81 (6H; t, $J=6.7$ Hz, $2CH_2CH_3$), 1.15–1.31 (40H; 2 m, $20CH_2$), 1.35–1.43 (4H; m, 2 x $(CH_2)_{10}CH_2CH_2C\equiv C$), 2.01–2.06 (4H; m, 2 x $(CH_2)_{11}CH_2C\equiv C$), 2.34–2.41 (4H; m, $2CH_2CH_2CO_2$), 2.42–2.49 (4H; m, $2CH_2CO_2$), 3.05–3.13 (m, 2H; CH_2NH_3), 3.85–3.91 (m, 2H; glycerol-C3-$H_{a,b}$), 3.97–4.05 (m, 2H; $CH_2CH_2NH_3$), 4.12 (1H; dd, $J=12.0$, 6.4 Hz, glycerol-C1-H_b), 4.33 (1H; dd, $J=11.8$, 2.6 Hz, glycerol-C1-H_a), 5.17 (1H; m, glycerol-C2-H), 8.20–8.60 (br s, NH_3); δ_C (100 MHz; $CDCl_3$) 14.5 (2 signals), 15.0, 15.1, 19.1, 23.1, 29.4, 29.4, 29.5, 29.5, 29.6, 29.7, 29.8, 30.0, 30.0, 30.1, 30.1, 32.3 (26 signals), 34.3, 34.4, 40.8, 62.6 (m) 63.1 (m), 64.2 (d, $J_{CP}=5.5$ Hz), 70.9 (d, $J_{CP}=6.9$ Hz), 78.1, 78.2, 81.6, 81.7, 172.0, 172.2; HRMS (FAB^+) calcd for $[C_{41}H_{74}NO_8P+H]^+$ 740.523033, found 740.523087; HPLC $t_R=4.3$ min.

Distear-9-ynoyl L-α-phosphatidylethanolamine (DS(9-yne)PE, 3): (211 mg, 94%); R_f 0.58 [solvent B]; ν_{max} (nujol mull) cm^{-1} 2219, 1709, 1431, 1274, 1072; δ_H (270 MHz; $CDCl_3$) 0.81 (6H; t, $J=6.8$ Hz, $2CH_2CH_3$), 1.15–1.59 (44H; 2 m, $22CH_2$), 2.03–2.13 (8H; m, $2CH_2C\equiv CCH_2$), 2.19–2.30 (4H; m, $2CH_2CO_2$), 3.09 (m, 2H; CH_2NH_3), 3.88 (m, 2H; glycerol-C3-$H_{a,b}$), 4.02 (m, 2H; $CH_2CH_2NH_3$), 4.07 (1H; dd, $J=$ 12.0, 6.8 Hz, glycerol-C1-H_b), 4.32 (1H; dd, $J=11.6$, 2.4 Hz, glycerol-C1-H_a), 5.15 (1H; m, glycerol-C2-H), 8.20–8.45 (br s, NH_3); δ_C (100 MHz; $CDCl_3$) 14.49 (2 signals), 19.1, 23.1, 25.2, 25.3, 29.2, 29.3, 29.4, 29.5, 29.5, 29.6, 29.6, 32.2 (26 signals), 34.4, 34.6, 40.8, 62.6 (m) 63.0 (m), 64.3 (d, $J_{CP}=4.0$ Hz), 70.7 (d, $J_{CP}=7.4$ Hz), 80.4, 80.7 (4 signals), 173.7, 173.5; HRMS (FAB^+) calcd for $[C_{41}H_{74}NO_8P+H]^+$ 740.523033, found 740.523073; HPLC $t_R=4.3$ min.

Distear-14-ynoyl L-α-phosphatidylethanolamine (DS(14-yne)PE, 4): (207 mg, 93%); R_f 0.56 [solvent B]; ν_{max} (nujol mull) cm^{-1} 2291, 1728, 1461, 1238, 1075; δ_H (270 MHz; $CDCl_3$) 0.90 (6H; t, $J=7.4$ Hz, $2CH_2CH_3$), 1.17–1.34 (34H; 2 m, $17CH_2$), 1.36–1.48 (6H; m, $3CH_2$), 1.48–1.56 (4H; m, $2CH_2$), 2.02–2.10 (8H; m, $2CH_2C\equiv CCH_2$), 2.18–2.28 (4H; m, $2CH_2CO_2$), 3.03–3.15 (m, 2H; CH_2NH_3), 3.82–3.93 (m, 2H; glycerol-C3-$H_{a,b}$), 3.97–4.05 (m, 2H; $CH_2CH_2NH_3$), 4.08 (1H; dd, $J=12.0$, 6.8 Hz, glycerol-C1-H_b), 4.31 (1H; dd, $J=11.8$, 2.6 Hz, glycerol-C1-H_a), 5.14 (1H; m, glycerol-C2-H), 8.20–8.60 (br s, NH_3); δ_C (100 MHz; $CDCl_3$) 13.9 (2 signals), 19.1, 22.9, 25.3, 25.3, 29.3, 29.6, 29.8, 29.8, 29.9, 30.00, 30.1, 30.1 (26 signals), 34. 5, 34.6, 40.8, 62.6 (m) 63.0 (m), 64.3 (m), 70.6 (d, $J_{CP}=8.0$ Hz), 80.4, 80.7 (4 signals), 173.5, 173.8; HRMS (FAB^+) calcd for $[C_{41}H_{74}NO_8P+H]^+$ 740.523033, found 740.522568; HPLC $t_R=4.3$ min.

Liposome preparation: A uniform lipid film was obtained by mixing lipid stock solutions in chloroform at 5 mg mL^- at the desired mol ratio of lipids followed by slow removal of solvent in vacuo. The film was subsequently hydrated with the appropriate amount of low conductivity (18.2 MΩ) water to a final concentration of either 0.5 mg mL^{-1} (in vitro studies) or 10 mg mL^{-1} (X-ray diffraction studies) and extensively sonicated to clarity at 50 °C to ensure uniform unilamellar vesicle formation.

In vitro cell studies: Panc-1 cells were cultured in DMEM (Dulbecco's Modified Eagle Medium, Gibco BRL) supplemented with 1% (*v/v*) penicillin-streptomycin (Gibco BRL) with or without 10% (*v/v*) fetal calf serum (FCS; Gibco BRL) at 37 °C in a humidified atmosphere with 10% CO_2. For efficiency studies, cells were seeded and subjected to transfection with an average of 50000 cells per well in 24-well plates resulting in a cells at 70–80% confluent. To prepare LD systems, precalculated amounts of liposome (0.5 mg mL^{-1}) and pEGFP-LUC DNA (1 mg mL^{-1}; BD Biosciences) were taken from their stock solutions and diluted with DMEM to a final volume of 125 μL. The DNA solution was then added to the liposome solution. The resultant LD particles contained pEGFP-LUC DNA (0.5 μg) with a lipid/DNA ratio of 12:1 (*w/w*), which were then added to cells. After 4 h transfection, cells were rinsed three times with PBS (phosphate buffered saline, Gibco BRL), and incubated in supplemented DMEM for an additional 24 h to allow expression of the luciferase gene. Results were quantified using the Promega Luciferase Assay System and a Berthold Luminometer (Lumat LB 9507). The BCA Protein Assay (Pierce) was used to determine the amount of total cellular protein for normalization. For cell viability, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) studies, Panc-1 cells were seeded and subject to transfection as described above. After the 24 h incubation time, a cell proliferation kit (MTT; Roche) assay was used to assess cell viability post-transfection.

Photon correlation spectroscopy: Particle sizes of the liposomes and lipoplexes were measured using dynamic light scattering (Coulter N4 plus). The detector was kept at a fixed angle of 90° and correlations were measured over 60 s in triplicate. Liposome concentrations were kept at approximately 0.5 mg mL^{-1} to obtain an adequate signal.

X-ray scattering

Small angle X-ray scattering (SAXS): Complexes for SAXS studies were prepared by mixing liposomes (10 mg mL^{-1}) with 0.2 mg of highly polymerized calf thymus DNA (5 mg mL^{-1}; Sigma) to a final lipid/DNA ratio of 12:1 (*w/w*). Data were obtained by using an in-house rotating copper anode source with Osmic™ multilayer optics and imaged with a MAR image plate detector.

Wide angle X-ray scattering (WAXS): Liposomes for WAXS studies were prepared in excess water conditions (>50%). Temperature increases were made over periods of 30 s, and in each case the sample was allowed to equilibrate for 5 min before each measurement. Data were obtained by using an in-house Bede Microsource™ generator equipped with an integrated focusing capillary

optic (XOS) and imaged with a Gemstar HS intensified CCD detector.

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