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Substitution of unsaturated lipid chains by thioether containing lipid chains in cationic amphiphiles: physico-chemical consequences and application for gene delivery.

Amal Bouraoui, Mathieu Berchel, Rosy Ghanem, Véronique Vié, Gilles Paboeuf, Laure Deschamps, Olivier Lozach, Tony Le Gall, Tristan Montier and Paul-Alain Jaffrès

The hydrophobic moiety of cationic amphiphiles plays an important role in the transfection process because its structure has an impact on both the type of the supramolecular assembly and the dynamic properties of these assemblies. These latter have to exhibit a compromise between stability and lability to efficiently compact then deliver DNA into target cells. In the present work, we report the synthesis of new cationic amphiphiles featuring a thioether function at different positions of two 18-atoms length lipid chains and we study their physicochemical properties (anisotropy of fluorescence, compression isotherms) with analogues possessing either oleyl (C18:1) or stearyl (C18:0) chains. We show that the fluidity of cationic lipids featuring a thioether function located close to the middle of each lipid chain is intermediate with that of oleyl- and stearyl-containing analogues. These properties are also supported by the compression isotherms assays. When used as carriers to deliver a plasmid DNA, thioether-containing cationic amphiphiles demonstrate a good ability to tranfect human-derived cell lines, those incorporating such a moiety in the middle of the chain being the most efficient. This work supports the use of a thioether function as a possible alternative to unsaturation in aliphatic lipid chains of cationic amphiphiles to modulate physicochemical behaviours and in turn biological activities such as gene delivery ability.

Introduction

Cationic amphiphilic compounds, formulated as a liposomal solution, have the ability to interact spontaneously with nucleic acids and to compact them into cationic nanoparticles (lipoplexes). These aggregates have the capacity to cross the cell plasma membrane and to deliver nucleic acids into the cytoplasm. Such nanoparticles demonstrated noticeable interests for different in vitro and in vivo applications including the transfection of cultured cells mimicking pathologic situations, lung transfection, vaccination or tendon healing. Synthetic carriers such as cationic lipids can be synthesized on large scale and according to the Good Manufacturing Practices (GMPs). Furthermore, their use for in vivo applications does not induce any immune response in animal and human hosts, thus permitting repeated administrations. In our group, we have developed straightforward syntheses (in 2 to 4 steps) of bio-inspired lipophosphoramidates, lipothiophosphoramidates or lipophosphates. Besides the polar headgroup and the linker, - the lipid moiety was identified as a crucial parameter determining the interaction with DNA. However, if DNA compaction is usually required for gene delivery, too much stable lipoplexes typically yield low transfection efficacies as observed for instance with amphiphiles incorporating stearyl (C18:0) lipid chains. The incorporation of unsaturation or ramification (phytanyl chains or synthetically branched lipid chains) within the hydrophobic moiety disturbs the packing of the lipid chains which in turn is correlated with low temperature fused lipid phase and higher transfection efficacies. It was suggested that a subtle balance between stability (needed before cell internalization) and instability (required after internalization by endocytosis) of the lipoplexes has to be reached. The lipid moiety plays a central role to tune this balance. One possibility consist to place an unsaturation within the lipid chains (e.g. oleyl chains). However, alkene of Z configuration may present some chemical instability. Therefore, it is worth exploring other hydrophobic domains that would be chemically stable and devoid of chirality to design new nucleic acid carriers.

Here, our attention focused on the incorporation of a thioether non polar functional group within the hydrophobic domain of cationic amphiphilic compounds. The use of thioether function was reported by Dobner et al. for the synthesis of bolaphospholipids featuring a central lipid chain where two methylene units were replaced with either two oxygen or two sulfur atoms. Molecular modelling pointed out that the incorporation of thioether groups induced two kinks in the lipid chains likely explained by the replacement of the all-trans...
conformation characterized by CCC bond angle of 113.3° by CSC bond angles of 96.1°. A second study, investigated the incorporation of thioether function within the structure of ionic liquids.16 It was shown that the replacement of one methylene unit of a C18:0 alkyl chain by a thioether function induced a drastic reduction of the melting point attesting important supramolecular modifications. Moreover, melting point values depended on the position of the thioether function in the alkyl chain underlying an impact at the supramolecular level. Therefore, the present study aims to design cationic amphiphilic compounds in which the Z-C=O double bond of the oleyl chain (which is likely the most used for the design of cationic amphiphiles used for nucleic acids delivery) is replaced by a thioether function within a straight (non-branched) hydrophobic domain. Additionally, taking into account the paper of O’Brien et al.16 that assessed the influence of the position of the thioether function on the physico-chemical properties, we report herein the synthesis of the compounds 4a-d (Figure 1) characterized with a thioether function at different positions in the lipid chains. Two additional cationic amphiphiles were included in this study for comparison purposes. They incorporated either two mono-unsaturated oleyl (C18:1) lipid chains (BSV36) or two saturated stearic (C18:0) alkyl chains (BSV101). The six compounds shown in Figure 1 thus featured exactly the same number of atoms (18 atoms) in their backbone lipid chains whereas the polar head group and the spacer are rigorously identical. This homogenous series of compounds permitted relevant comparisons of both physico-chemical properties and transfection activities allowing to clearly address the potential impact of thioether function within lipid chains of cationic amphiphiles.

Results and discussion

The synthesis of compounds 4a-d starts by the synthesis of the dialkyl phosphites possessing in ω-position of the alkyl chain one unsaturation (Figure 2). These compounds 1a-d were prepared according to two methods. The first one is based on the reaction of allyl alcohol or pent-4-en-1-ol with PCl₃ followed by a hydrolysis step.17 The second method, that requires a non-volatile alcohol, is based on the reaction of alcohol with diphenylphosphite under reduced pressure which is needed to eliminate phenol (a side-product of this reaction) by sublimation. These phosphites 1a-d were then engaged in an Atherton-Todd’s reaction18 with N,N-dimethylethylenediamine to produce the phosphoramidic 2a-d. The third step consisted in the quaternarization of these compounds with methyl iodide to produce, in good yields, the cationic amphiphiles 3a-d. The last step, make use of the photo-click thiol-ene reaction triggered by UV and using 2,2-DiMethoxy-2-PhenylAcetophenone (DMPA) as radical initiator. Accordingly, alkyl thiols of different lengths were selected to produce at the end the cationic amphiphiles 4a-d each possessing 18 atoms in their backbone lipid chains (spectroscopic data of compounds 4a-d are available in ESI 1).

The Critical Aggregation Concentration (CAC) of compound 4a-d was determined by fluorescence (using the Nile red as fluorescent probe19). Compounds 4a-d feature similar value (around 1 to 2×10⁻⁵ mol/L) as for BSV101 and BSV36 (ESI 2).

Then, all the compounds were formulated as a liposomal solution at 1.5 mM in water by using the lipid film hydration method. Interestingly, we found that it was possible to prepare liposomal solutions with these new cationic amphiphiles after a
These experiments give information relative to the propensity of the cationic amphiphiles to cover water surface and the pressure needed to compact the amphiphiles up to the collapse that corresponds to the destruction of a monolayer. Since all the compounds possess an identical polar head group, the difference of behaviour arises only from the different structures of the hydrophobic domain. The minimal mean molecular area ($\text{Å}^2$/molecule) corresponding to pressure of 0 mN/m (lift-off values noted $A_0$) is a first indication of the supramolecular organization of the hydrophobic domain (Table 1). In this regard, BSV101 (featuring saturated alkyl chains) exhibits the lower $A_0$ value (80 $\text{Å}^2$/molecule) that can likely be explained by the fact that the alkyl chains are partly in all-trans conformation, thus reducing the surface area. The addition of one unsaturation (BSV36) or a thioether function (4a-d) increases the $A_0$ value from 110 (4b) to 180 (4c and 4d) $\text{Å}^2$/molecule in comparison with BSV101. The compounds 4c, 4d and BSV36 are those featuring the highest $A_0$ value and can therefore be considered as the compounds with the most disordered lipid chains in absence of applied pressure. The second significant point is the molecular area at the collapse pressure that indicates how the molecules can be packed together before producing interfacial multi-lamellar packing or aggregates diving in the water phase. To this respect, the presence of unsaturations (BSV36) is a structural feature that induces a collapse at higher mean molecular area indicating that the volume occupied by the oleyl chains is higher than for the other lipid chains considered herein. On the other hand, the compounds 4a-d feature a small surface area at the collapse (from 40 (4b) to 60 (4c) $\text{Å}^2$/molecule) indicating that the volume of the lipid domain is smaller and/or that these lipid chains can produce a regular packing leading to a small surface area. It can be noted that in absence of pressure compounds 4c-d occupy high surface area whereas in presence of pressure they can self-organized in a dense packing pointing out a capacity to adapt their conformation depending on the constrains. Finally, the pressure at the collapse is an indication of the robustness of the supramolecular packing. In this regard, the absence of unsaturation or thioether function close to the middle of the lipid chain produces the most robust supramolecular packing (4a and BSV101; 50 and 58 mN/m). All the other compounds featured a lower value for the pressure at the collapse at 47 mN/m (Table 1) indicating that the robustness of the monolayer is globally identical for BSV36, 4b, 4c and 4d. Simultaneously to the surface ellipsometric angle ($\Delta$, °) was recorded during the compression isotherms using a home-made ellipsometer, in the so-called “null-ellipsometer configuration”. The ellipsometric angle ($\Delta$) depends on two parameters, the reflexion index and the thickness of the lipid film. The reflexion index is considered constant during compression permitting to consider $\Delta$ as a function of the film thickness. As shown in the Supporting Materials (ESI 4), in absence of pressure the compounds 4c and 4d produced the thinner monolayer (lower value of $\Delta$) whereas BSV101 produced the thicker monolayer. These results are fully consistent with the lift-off values (Table 1).

![Figure 3 Compression isotherms for compounds 4a-d and compounds BSV101 and BSV36.](image-url)

<table>
<thead>
<tr>
<th></th>
<th>Lift-off value - $A_0$</th>
<th>Surface area at the collapse</th>
<th>Pressure at the collapse</th>
</tr>
</thead>
<tbody>
<tr>
<td>4a</td>
<td>140</td>
<td>50</td>
<td>50</td>
</tr>
<tr>
<td>4b</td>
<td>110</td>
<td>60</td>
<td>47</td>
</tr>
<tr>
<td>4c</td>
<td>180</td>
<td>45</td>
<td>47</td>
</tr>
<tr>
<td>4d</td>
<td>180</td>
<td>45</td>
<td>47</td>
</tr>
<tr>
<td>BSV36</td>
<td>160</td>
<td>75</td>
<td>47</td>
</tr>
<tr>
<td>BSV101</td>
<td>80</td>
<td>45</td>
<td>58</td>
</tr>
</tbody>
</table>

$A_0$: Minimal molecular surface area at a pressure of 0 mN/m (lift-off values)
Then, fluorescence anisotropy was used to probe the viscosity of the supramolecular packing of amphiphilic compounds. For that purpose, diphenylhexatriene (DPH) was used as a hydrophobic fluorescent probe. This probe is known to be localized within the hydrophobic region and inform on the dynamic process as previously reported for other amphiphilic compounds. As reported in Figure 4, the structure of the lipid chain deeply impacts their supramolecular dynamics. Indeed, the saturated C18 lipid chain (BSV101) features a transition from gel to fluid phase at the highest temperature (50°C). This observation is also in agreement with the pressure at the collapse, which was the more intense for BSV101 (Table 1). The incorporation of a thioether function decreases the values of the main transition (Tm) with a great influence of its position within the lipid chain. Indeed, compound 4a features a transition at 35°C whereas the other three compounds 4b-d feature almost a similar Tm between 16 to 19°C. These results are again consistent with the pressure at the collapse since the more robust monolayer (4a, BSV101 ; Table 1) features the highest Tm. Finally, the presence of one unsaturation per lipid chain or a thioether group indicates that the Tm is even lower in presence of an oleyl chain (BSV36 ; Tm <0°C). However, at physiological temperature (37°C), the thioether containing amphiphiles 4b-d and the unsaturated compound BSV36 feature almost similar anisotropy. The fluidity of the compounds 4a-d is likely due to the presence of the sulfur atom that prevents a close packing of the molecules. The bigger size of sulfur atom and the induction of a bend in the hydrophobic chain due to the bond angle and bond length involving the sulfur atom are the two parameters that likely account for the fluidity at lower temperature. This bend was determined using molecular modeling. As detailed in the supporting material (ESI 5), a bend of 175.6° was estimated by molecular modelling for the lipid chains present in molecules 4a-d. Next, we recorded for one sample (4d) 31P NMR (Hahn echo sequence) spectra to further study the supramolecular organization. It must be noted that the reference compound BSV36 produced in similar hydration state a layered packing. The 31P NMR spectrum of compound 4d (hydrated at a concentration of 100 mg/mL) indicates that this compound adopts also a layered supramolecular packing as attested by both the symmetry of the signal (the most intense peak ω₁ is on the right hand side of the pattern) and the chemical shift anisotropy (Δσ > 40 ppm) (ESI 6).

Then, the capacity of compounds 4a-d to transfect two human-derived cell lines (i.e. A549 and 16HBE) was assessed at different charge ratios (CR); the latter corresponds to the number of positive charges belonging to the cationic amphiphiles divided by the number of negatives charges coming from the nucleic acids. Since the best transfection efficacies were generally measured at CR=4 (see the full data in the ESI 8), the transfection efficacies at that CR for compounds 4a-d and the two references (BSV101, BSV36) are provided for comparison in figure 5 (the reference compound BSV36 used in this series of assays was previously compared to Lipofectamine). In agreement with previous investigations, saturated alkyl chains (BSV101) are detrimental to transfection efficacies likely due to a too robust supramolecular packing as shown by compression isotherms and fluorescence anisotropy. For A549 cell line, the transfection assays were repeated with a lower quantities of plasmid (0.1 µg/well) and the trend was similar since the best transfection efficacies were obtained at CR=4 and the most efficient cationic amphiphiles were compounds 4c and 4d (ESI 7). The incorporation of a thioether function within the lipid chain can have a positive impact on the transfection efficacies, depending on the position of this function within the hydrophobic chain. A thioether group close to the polar head group (phosphoramide function) has only a weak positive effect on the transfection efficacy (compounds 4a and 4b). On contrary, a thioether function in the middle of the hydrophobic domain (at position 10 or 12) produces efficient gene carriers (compounds 4c and 4d). On contrary, a thioether function in the middle of the hydrophobic domain (at position 10 or 12) produces efficient gene carriers (compounds 4c and 4d). Interestingly, cationic amphiphiles bearing thioether-containing lipid chains yield similar or even better efficacies (depending on the cell line considered) when compared with structural analogues featuring unsaturated lipid chains (i.e. oleyl chains, which are present in BSV36 as well as in a large
number of cationic amphiphiles commonly used for gene delivery purposes). It is interesting to notice that the most efficient compounds 4c-d combined three identical indicators (Tm below 20°C; pressure at the collapse below 48 mN/m; lift-off pressure A0 > 150 mN/m). Then, the cell viability was assessed by a bioluminescent method (quantification of ATP) or MTT assays (ESI 8). 80% of viability was globally observed at CR=4 (the most efficient CR) and a higher tolerance was observed for compound 4d (ESI 8). Altogether, we can conclude that the thioether containing amphiphiles, and especially compound 4d, have a weak side effect on the viability and that this viability is similar to those observed with BSV36 (cationic amphiphiles possessing two oleyl chains). Noteworthy, all these experiments (transfection and viability) were carried out with the cationic amphiphiles formulated alone. The incorporation of co-lipids within the formulations or the use of other strategies recently reported could reduce the side toxic effects.23

These results show that the incorporation of thioether function within the lipid chain is an interesting strategy to produce gene carriers. The two most efficient carriers reported herein (4c and 4d) have a thioether function located close to the middle of each lipid chain.

**Experimental**

**A Material**

All commercial reagents were used as received. Solvent were dried with a MBRAUN Solvent Purification Systems (SPS-800 series). 1H, 13C, 31P NMR were recorded on Bruker spectrometers (300, 400 or 500 MHz). Chemical shift δ are express in ppm and the abbreviation used are s (singlet), d (doublet), t (triplet), q (quadruplet). Mass spectra was recorded on Bruker MALDI-TOF autoflex III ; 2,5-Dihydroxybenzoic acid (DHB) was used as matrix. Fluorescence was recorded on Agilent Cary Eclipse Fluorescence Spectrophotometer, using automatic polarizers for anisotropy measurements. Size and zeta measurement were achieved with a MALVERN Nano ZS. BSV36 and BSV101 were syntheses as previously reported.24

**B Synthesis.**

**Synthesis of compounds 1a and 1b:** (the protocol was adapted from T. W. Kassa et al.,) in a 500 mL two neck round bottom flask fitted with an addition funnel, phosphorus trichloride (1 equivalent) was dissolved in 150 mL of dry toluene and cooled at 10°C. A mixture containing two equivalents of either allyl alcohol (for the synthesis of 1a) or pent-4-ene-1-ol (for the synthesis of 1b) and triethylamine (2 eq.) was added slowly over a period of 1 h. under vigorous stirring. The resulting mixture was allowed to warm to room temperature (rt) and one additional equivalent of either allyl alcohol or pent-4-ene-1-ol was added. Then the reaction was stirred overnight at rt. Triethylamine hydrochloride precipitate was removed by filtration over celite and washed several times with toluene. The organic layer was washed with water (100 mL) and then dried over Na2SO4, filtered and concentrated to give a colorless oil.

1a (x g ; Yield 97%): 1H NMR (400 MHz, CDCl3): δ = 4.48-4.51 (m, 4H), 5.17-5.32 (m, 4H), 5.81-5.85 (m-2H), 5.92-7.68 (d, JHP 703 Hz, 1H); 31P{1H} NMR (162 MHz, CDCl3): δ = 8.3; 13C{1H} NMR (75 MHz, CDCl3): δ = 65.2, 117.5, 130.4.

1b (x g ; Yield 98%): 1H NMR (400 MHz, CDCl3): δ = 1.71-1.75 (m, 4H), 2.05-2.11 (m,4H), 3.97-4.03 (m-4H), 4.91-4.99 (dd, 4H), 5.67-5.74 (m, 2H), 5.86-7.59 (d, JHP 692 Hz, 1H); 31P{1H} NMR (CDCl3, 162 MHz): δ = 8.4; 13C{1H} NMR (75 MHz, CDCl3): δ = 28.29-29.07, 66.47, 115.16, 136.53.

**Synthesis of compound 1c-d:** non-8-en-1-ol (undecen-1-ol) (2 eq.) and diphenyl phosphite (1 eq.) were combined and treated with Kugelroth distillation under reduced pressure for 3 h at 130°C to remove propanol. The expected product, that doesn’t distill, was obtained as colorless oil.

1c (x g ; Yield 91%): 1H NMR (400 MHz, CDCl3): δ = 1.39-1.48 (m, 16H), 1.75-1.79 (m, 4H), 2.01-2.13 (m, 4H), 4.12-4.18 (m, 4H), 4.99-5.10 (m, 4H), 5.83-5.93 (m-2H), 6.02-7.75 (d, JHP 692 Hz); 31P{1H} NMR (162 MHz, CDCl3): δ = 8.4.

1d (x g ; Yield 96%): 1H NMR (400 MHz, CDCl3): δ = 1.24-1.33 (m, 24H), 1.62-1.66 (m, 4H), 1.96-2.02 (m, 4H), 4.01-4.05 (m, 4H), 4.83-4.97 (m, 4H), 5.71-5.81 (m-2H), 5.89-7.63 (d, JHP 695 Hz); 31P{1H} NMR (162 MHz, CDCl3): δ = 8.4.

**General protocol for the synthesis of compounds 2a-d:** To a solution of compound 1 (1 eq.; 2.56x10-3 mol) in 20 mL CH2Cl2 was added N,N-Dimethylhydroxylamine (1.1 eq), DIPEA (1.1 eq) and BrCl (1.1 eq) at 0°C. The reaction was stirred for 15 min. at 0°C then for 4 h. at rt. The solvent was evaporated and 30 mL of diethyl ether were added and the mixture was stirred for 10 min. then filtered, and the solvent evaporated. The residue was dissolved in 20 mL of CH2Cl2, washed with water, dried over MgSO4 filtered and concentrated. The compound was purified by silica gel chromatography using CH2Cl2/MeOH: 90/10 (v/v) as eluant to produce a colorless oil.

2a (Yield 40%): 1H NMR (400 MHz, CDCl3): δ = 2.46 (s, 6H), 2.73 (t, J=6.0 Hz, 2H), 3.09-3.12 (m, 2H), 4.04-4.07 (m, 1H), 4.46-4.50 (m, 4H), 5.87-5.95 (m, 2H); 31P{1H} NMR (CDCl3, 162 MHz) δ = 10.1.

2b (Yield 52%): 1H NMR (400 MHz, CDCl3): δ = 1.63-1.67 (m, 4H), 1.99-2.04 (m,4H), 2.09 (s, 6H), 2.59 (t, J= 6 Hz, 2H), 2.81-2.85 (m, 2H), 3.34-3.37 (m, 1H), 3.84-3.87 (m, 4H), 4.83-4.92 (dd, 4H), 5.61-5.71 (m, 2H); 31P{1H} NMR (162 MHz; CDCl3) δ = 10.2.

2c (Yield 60%): 1H NMR (400 MHz, CDCl3): δ = 1.24-1.31 (m, 16H), 1.58-1.61 (m, 4H), 1.94-1.98 (m, 4H), 2.16 (s, 6H), 2.32 (t, J= 6.2 Hz, 2H), 2.87-2.95 (m, 2H) 3.27-3.31 (m, 1H), 3.89-3.98 (m, 4H), 4.84-4.94 (m, 4H), 5.68-5.76 (m, 2H); 31P{1H} NMR (162 MHz; CDCl3) δ = 9.9.

2d (Yield 72%): 1H NMR (400 MHz, CDCl3): δ = 1.11-1.20 (m, 24H), 1.45-1.51 (m, 4H), 1.83-1.88 (m, 4H), 2.04 (s, 6H), 2.21 (t, J=6.0 Hz, 2H), 2.75-2.82 (m, 2H), 3.31-3.37 (m, 1H), 3.78-3.81 (m, 4H), 4.72-4.83 (m, 4H), 5.56-5.62 (m, 2H); 31P{1H} NMR (CDCl3, 162 MHz) δ = 10.2.

**General synthesis of compound 3a-d:** to a solution of 2 (1 eq.; 2x10-3 mol) in CH2Cl2 (20 mL) was added methyl iodide (4 eq.) and the reaction was stirred overnight at rt. The solvent and the excess of methyl iodide were evaporated to give a clear yellow salt.

3a (Yield 92%): 1H NMR (400 MHz, CDCl3): δ = 3.42 (s, 9H), 3.44-3.52 (m, 2H), 3.79 (t, J=6.0 Hz, 2H), 4.46-4.49 (m, 4H), 4.81-4.95
**General protocol for the synthesis of compounds 4a-d:**

**compound 3 (1 eq, 1.6x10^{-3} mol) alkane thiol (4 eq.) and 2,2-dimethoxy-2-phenylacetophenone (0.4 eq.) were placed in a schlenk tube and degassed under argon for 10 min. The mixture was then placed under UV lights at rt for at least 4 h. The product was purified with silica gel chromatography using CH3Cl/MeOH: 90:10 (v/v) as eluent phase to give the desired compounds as a yellow salt.

**4a (BSV-S4 ; yield 58%):** ^1^H NMR (400 MHz, CDCl3): δ=0.85 (t; J= 6.8 Hz, 6H), 1.23-1.34 (m, 48H), 1.30-1.34 (m, 48H), 1.51-1.55 (m, 4H), 1.71-1.80 (m, 2H), 2.48 (t; J= 7.2 Hz, 4H), 2.58 (t; J= 7.2 Hz, 4H), 3.44 (s, 9H), 3.54-3.55 (m, 2H), 3.83 (t; J= 5.4 Hz, 4H), 4.07-4.11(m, 4H), 4.62-4.67 (m, 1H); ^31^P(^1^H) NMR (CDCl3 162 MHz) δ= 8.8; ^13^C(^1^H) NMR (CDCl3, 75 MHz): δ=13.6 (CH3-CH2), 22.1-31.6 (CH2 fatty chain, S-CH2), 35.6 (CH2-NH-P), 54.3 (CH3-N′), 65.0 (CH3-N′), 66.2 (d, CH2-O-P); MALDI-TOF (matrix: DHB): [M]^+ calculated for C36H64N2O12P2S2 = 723.566; observed [M]^+ = 723.667.

**4b (BSV-S6 ; yield 62%):** ^1^H NMR (400 MHz, CDCl3): δ=0.86 (t; J=7 Hz, CH3-CH2, 6H), 1.26-1.35 (m, CH2, 40H), 1.52-1.59 (m, 8H), 1.63-1.66 (m, 4H), 2.48 (t; J= 7.0 Hz, CH2-S, 8H), 3.46 (s, 9H), 3.56 (m, 2H), 3.94-3.98 (m, 2H), 4.37-4.40 (m, 1H); ^31^P(^1^H) NMR (CDCl3, 162 MHz) δ= 8.9; ^13^C(^1^H) NMR (CDCl3, 75 MHz): δ=13.6 (CH3-CH2), 22.1-31.7 (CH2 fatty chain, S-CH2), 35.6 (CH2-NH-P), 54.3 (CH3-N′), 66.2 (CH3-N′), 66.4 (d, CH2-O-P); MALDI-TOF (matrix: DHB): [M]^+ calculated for C36H64N2O12P2S2 = 723.566; observed [M]^+ = 723.708.

**4c (BSV-S10 ; yield 48%):** ^1^H NMR (400 MHz, CDCl3): 0.85 (t; J=6.2 Hz, 6H), 1.36 (m, 40H), 1.45-1.49 (m, 4H), 1.51-1.53(m, 4H), 1.55-1.60 (m, 4H), 1.62-1.69 (m, 4H), 2.46-2.52 (m, 8H), 3.46 (s, 9H), 3.54-3.55 (m, 2H), 3.85 (t; J = 5.6 Hz, 2H), 3.96-4.01 (m, 4H), 4.47-5.00 (m, 1H); ^31^P(^1^H) NMR (CDCl3, 162 MHz) δ= 8.8; ^13^C(^1^H) NMR (CDCl3, 75 MHz): δ=13.6 (CH3-CH2), 22.1-31.7 (CH2 fatty chain, S-CH2), 35.6 (CH2-NH-P), 54.3 (CH3-N′), 66.2 (CH3-N′), 66.7 (d, CH2-O-P); MALDI-TOF (matrix: DHB): [M]^+ calculated for C36H64N2O12P2S2 = 723.566; observed [M]^+ = 723.703.

**4d (BSV-S12 ; yield 55%):** ^1^H NMR (400 MHz, CDCl3): δ=0.86 (t; J=7 Hz, 6H), 1.26-1.35 (m, 4H), 1.52-1.59 (m, 8H), 1.63-1.66 (m, 4H), 2.48 (t; J= 7.4 Hz, 8H), 3.46 (s, 9H), 3.56 (m, 2H), 3.94-3.98 (m, 2H), 4.37-4.40 (m, 1H), ^31^P(^1^H) NMR (CDCl3, 162 MHz) δ= 8.9; ^13^C(^1^H) NMR (CDCl3, 75 MHz): δ=13.6 (CH3-CH2), 22.18-31.7 (CH2 fatty chain, S-CH2), 35.6 (CH2-NH-P), 54.4 (CH3-N′), 66.2 (CH2-N′), 66.7 (d, CH2-O-P); MALDI-TOF (matrix: DHB): [M]^+ calculated for C36H64N2O12P2S2 = 723.566; observed [M]^+ = 723.723.

**8 Compression isotherms and ellipsometry.**

The Langmuir trough was vigorously cleaned with ethanol and water then it was filled with the sub-phase (water). A good baseline in the (n-A) isotherm indicated the cleanliness of the interface. The cationic lipid in CHCl3/MeOH (2/1) mixture (25 μL) was slowly spread at the air-water interface using a Hamilton microlitre syringe. After evaporation of the solvents, the isotherm of the lipid monolayer was measured by compressing the barriers at the rate of 5 cm²/min. The surface pressure was measured using a filter paper held by a Wilhelmy balance connected to a microelectronic feedback system. The experiment was stopped once the collapse was reached. Simultaneously, the ellipsometric angle was recorded during the compression. The values of Lambda (ΔN) were reported in ES18. ΔN correspond to the value obtained at the lift off of the molecule (when the surface pressure began to increase) while ΔNmax correspond to the collapse point.

**B. CMC and anisotropy.**

The fluorescent probe 1,6-diphenylhexatriene (DPH) was used for anisotropy measurements whereas the solvatochromatic fluorescent probe Nile red was used for CMC determination. Further details are present in ES19.

**C. Transfection efficacy and cell viability.**

**DNA complexation:** Lipoplexes were prepared by mixing pDNA (pEFGP-Luc, Clontech) with each liposomal solution in Opti-MEM (Gibco). Addition of luciferase encoding pGM144 (0.25 μg/well) to the liposomal solutions was performed at concentrations corresponding to CR.

**Transfection efficiencies:** The in vitro reporter gene assay via the luciferase measurement was carried out as reported previously.

**Cell viability:** The cell viability was considered to estimate the toxicity resulting from the exposure of the cells to the lipoplexes and the transfection process. For 16HBE cell line we used the ViaLight kit (Lonza) was used to determine the ATP content which reflects the number of cells (transfected or not) in culture, as a result of both cell proliferation and cell mortality.
chains increase the fluidity of the supramolecular assemblies of thioether function close to the middle of the hydrophobic chains. The comparison of the compression isotherms, and variation point arises from the presence of a thioether function might include comments relevant to but not central to the matter under discussion, limited experimental and spectral data, and crystallographic data.

Conclusions
This study reports a simple procedure to incorporate in selective position of a hydrophobic chain a thioether function using photo-click thiol-ene coupling. Accordingly, a homogeneous series of compounds was prepared since the only variation point arises from the presence of a thioether function at different position. These four new compounds were compared with related compounds exhibiting either mono-unsaturated (oleyl chain) or fully saturated (C18-alkyl) lipid chains. The comparison of the compression isotherms, and fluorescence anisotropy led to the conclusion that the presence of thioether function close to the middle of the hydrophobic chains increase the fluidity of the supramolecular assemblies that can be interpreted as an increase of the disordered state of the lipid chains. The cationic amphiphiles 4c and 4d applied for gene delivery feature high transfection efficacies. The viability at the most efficient charge ratio is around 80% for both cationic amphiphiles indicating that further studies (e.g. association with helper lipid) will be needed to reduce toxicity. Nevertheless, the intrinsic physico-chemical features induced by the incorporation of thioether function in the structure of the lipid chain constitute a new possibility to tune the fluidity of supramolecular assemblies and for the design of other type of amphiphiles (zwitterionic, bola or anionic amphiphiles).

Conflicts of interest
There are no conflicts to declare.

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Notes and references
† Footnotes relating to the main text should appear here. These might include comments relevant to but not central to the matter under discussion, limited experimental and spectral data, and crystallographic data.