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

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1. NMR spectra

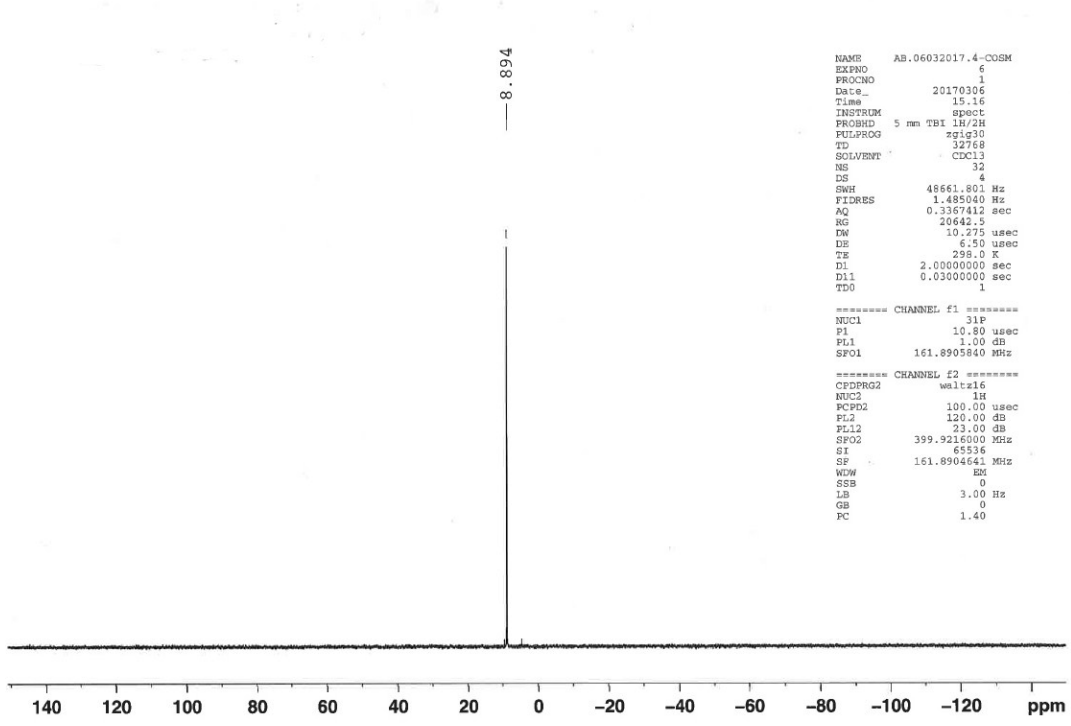


Figure SI1-1 : $^{31}P\{^{1}H\}$ NMR ($CDCl_3$) of compound **4a**

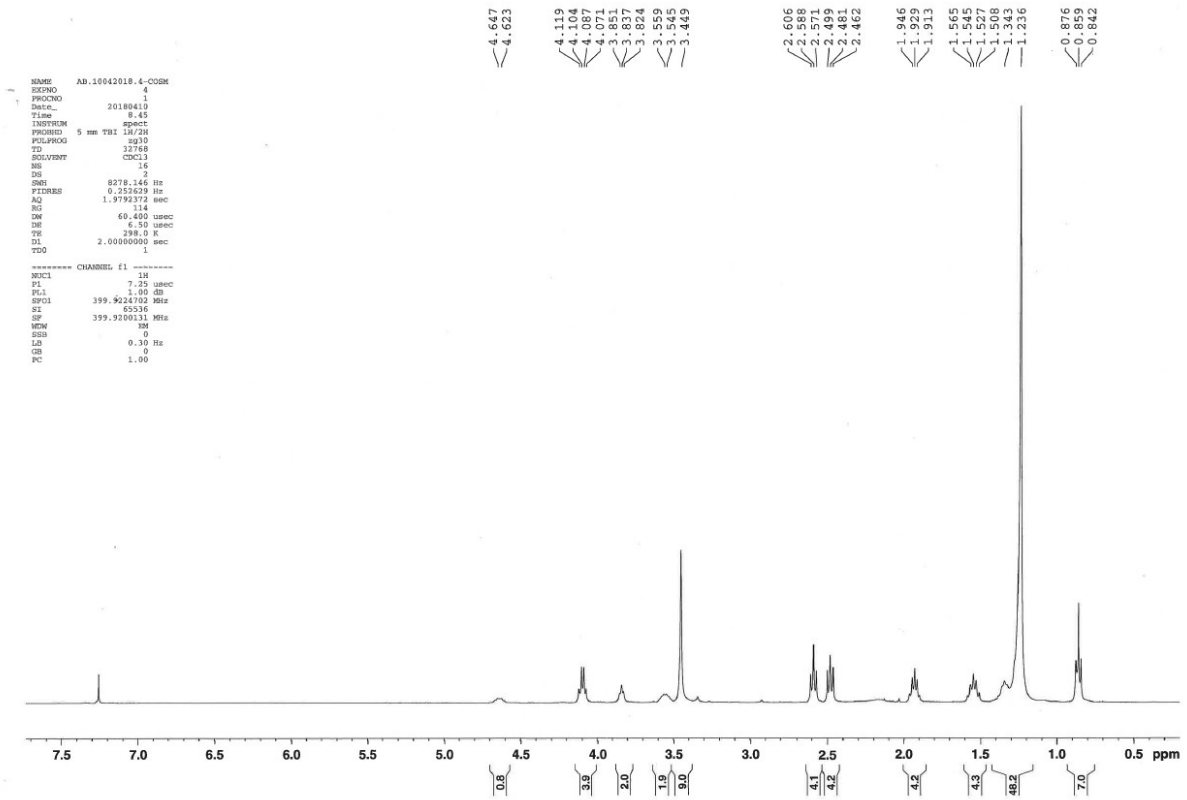


Figure SI1-2 : ^{1}H NMR ($CDCl_3$) for compound **4a**

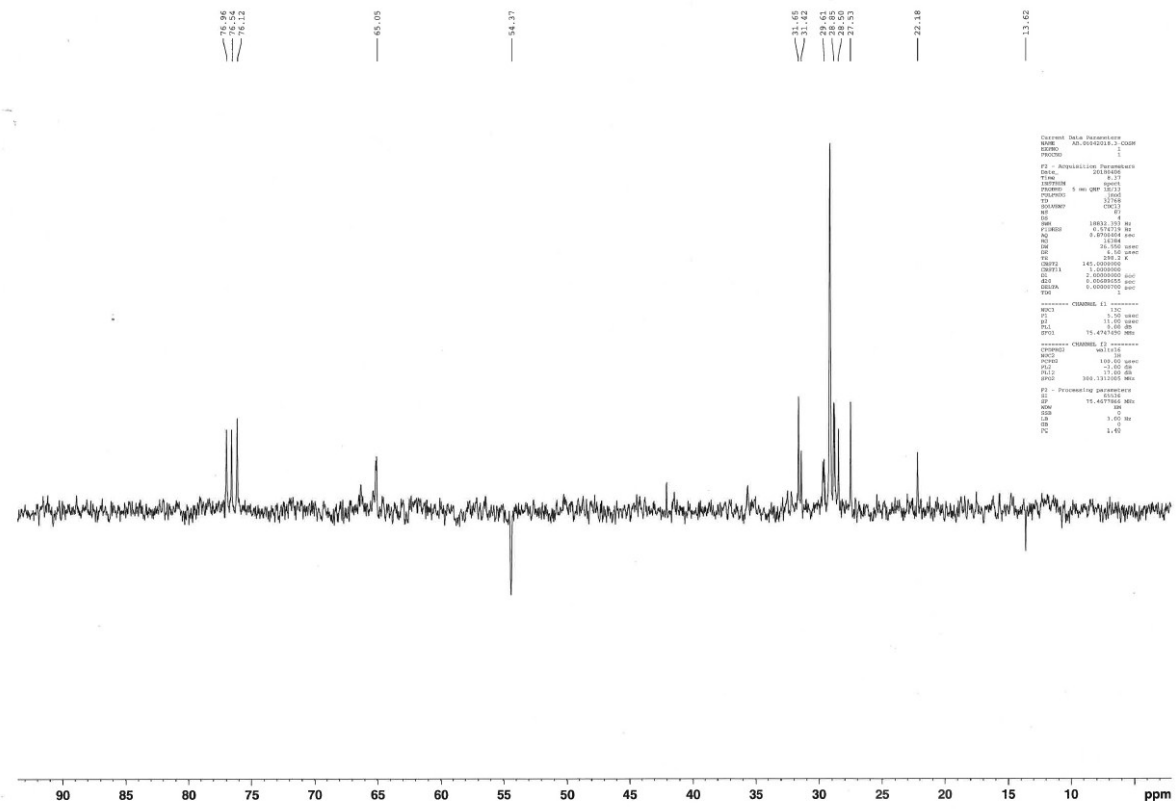


Figure SI1-3 : $^{13}C\{^{1}H\}$ NMR (Jmod ; $CDCl_3$) for compound **4a**

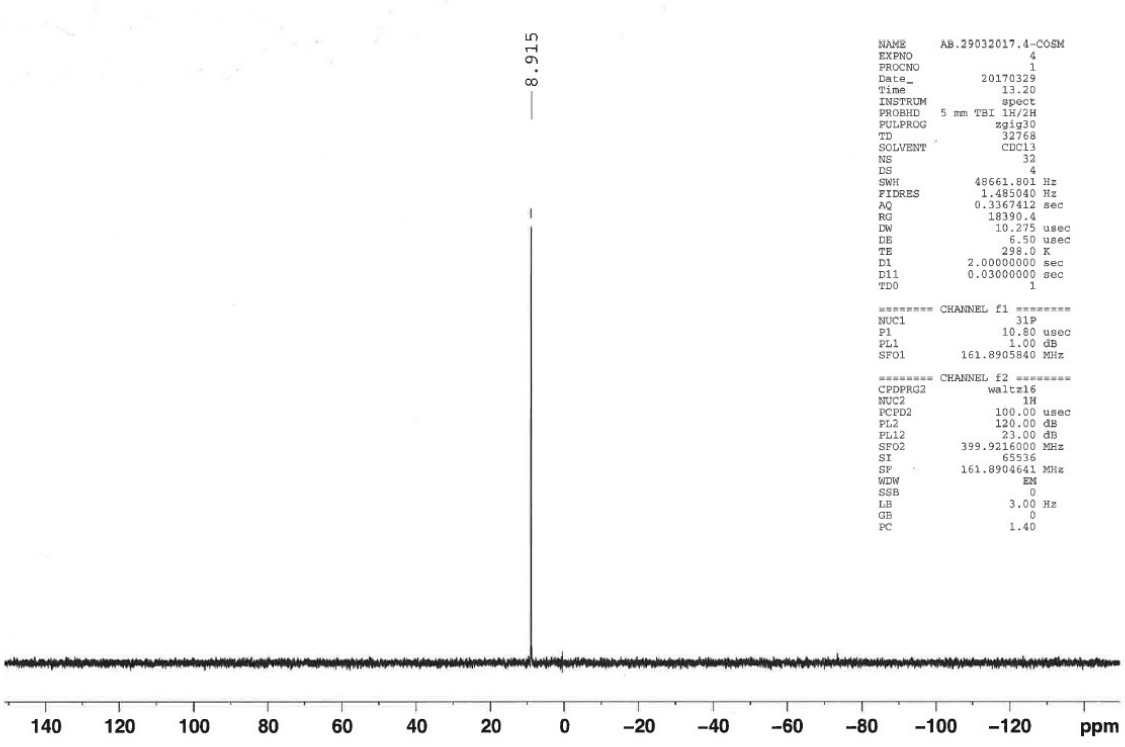


Figure SI1-4 : $^{31}P\{^{1}H\}$ NMR ($CDCl_3$) of compound **4b**

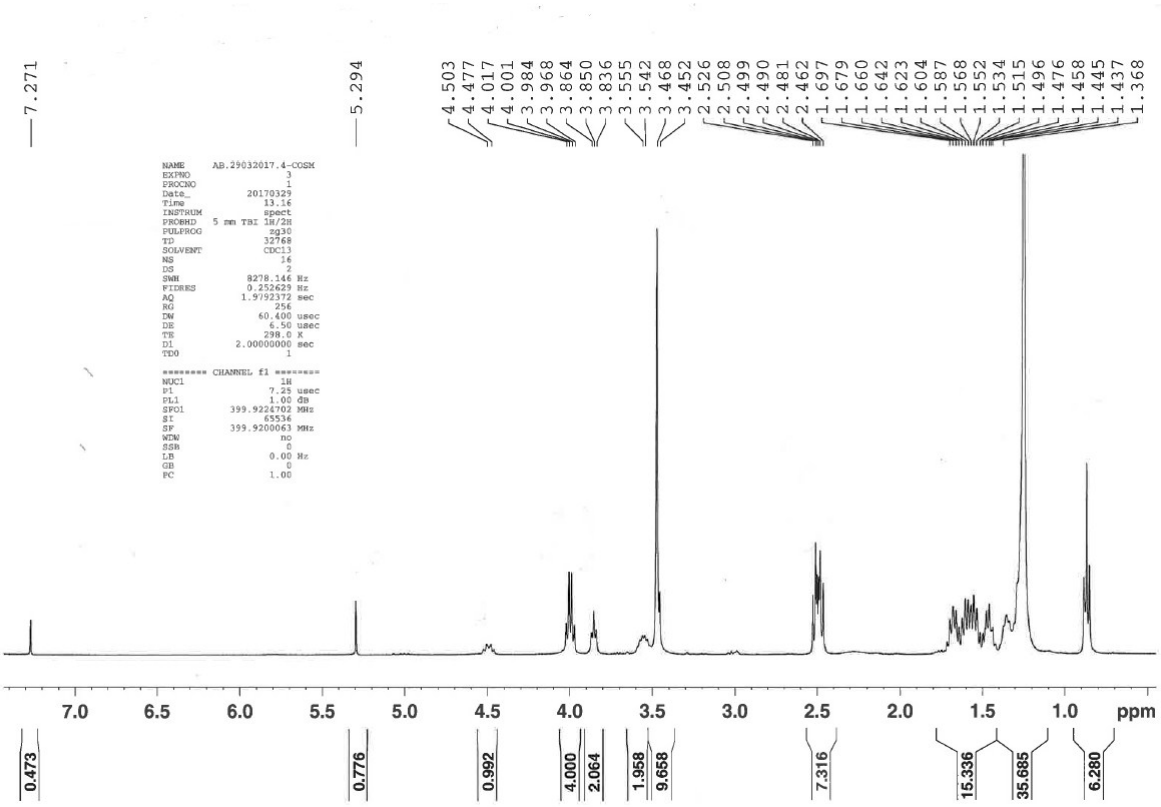


Figure SI1-5 : ^{1}H NMR ($CDCl_3$) for compound **4b**

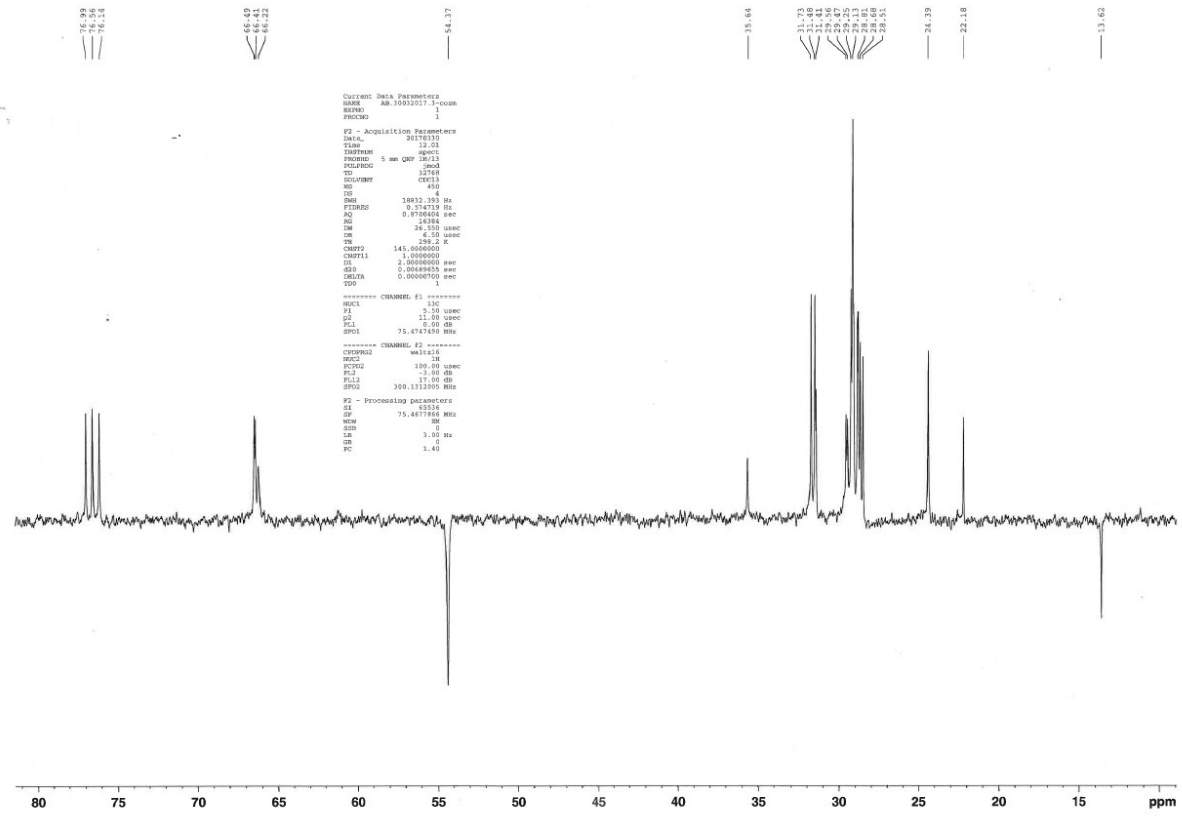


Figure SI1-6 : $^{13}C\{^{1}H\}$ NMR (Jmod; $CDCl_3$) for compound **4b**

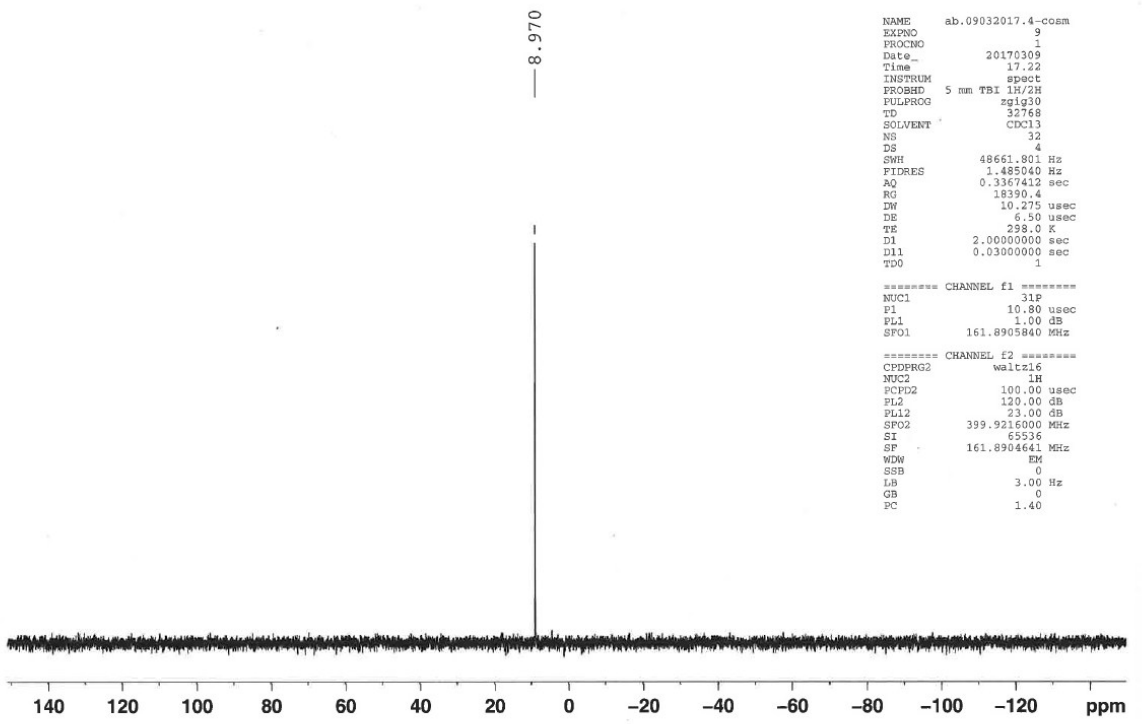


Figure SI1-7 : $^{31}P\{^{1}H\}$ NMR ($CDCl_3$) of compound **4c**

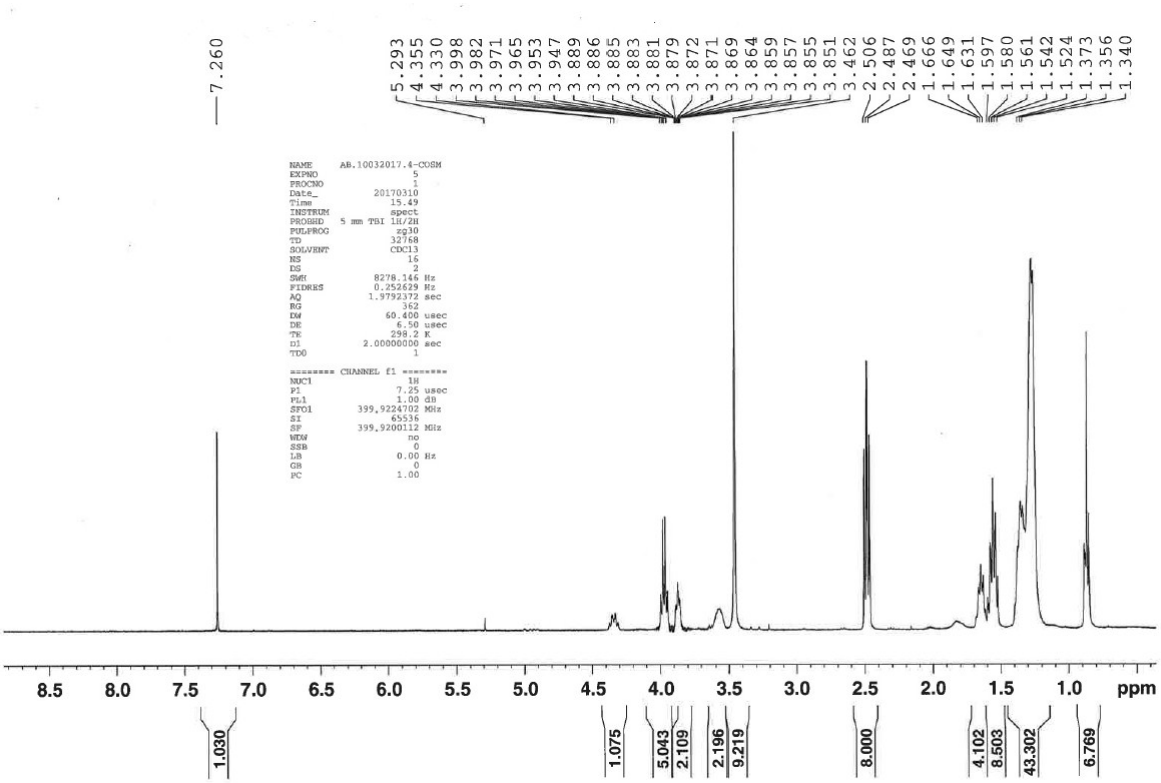


Figure SI1-8 : ^{1}H NMR ($CDCl_3$) for compound **4c**

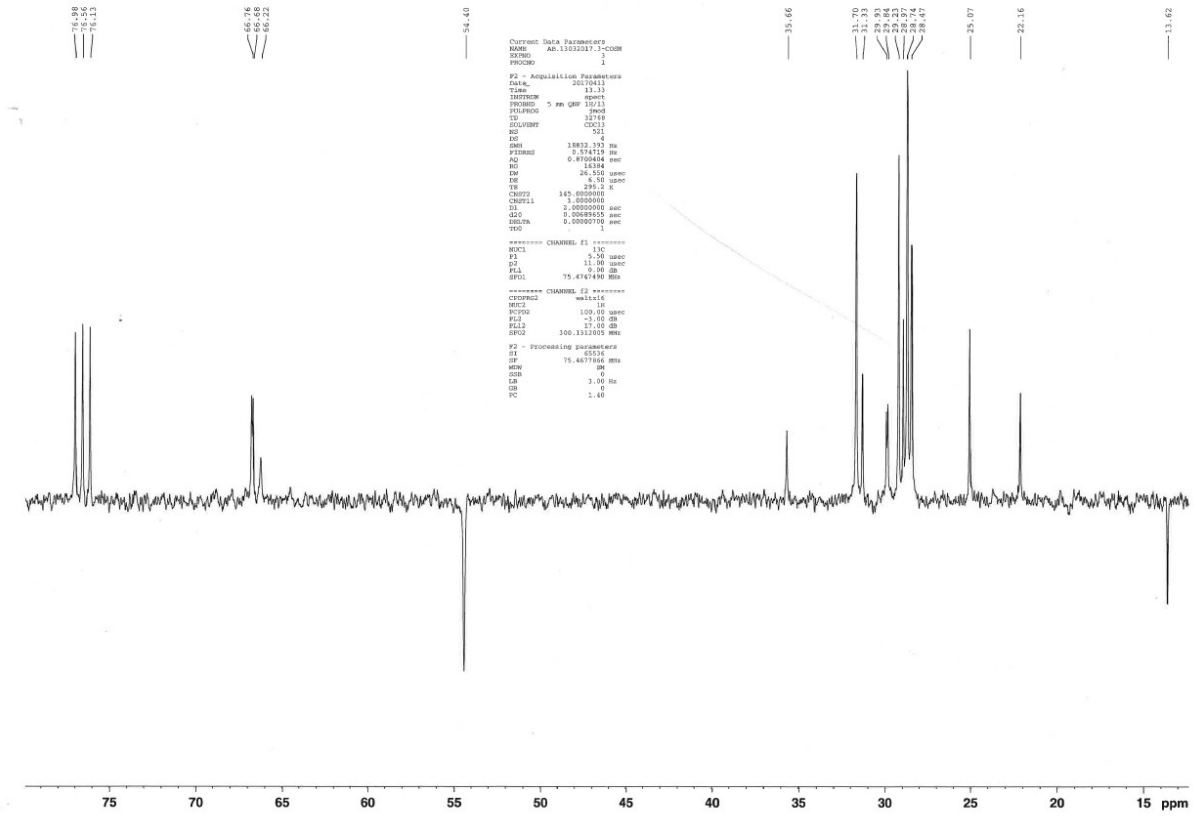


Figure SI1-9 : $^{13}C\{^{1}H\}$ NMR (Jmod; $CDCl_3$) for compound **4c**

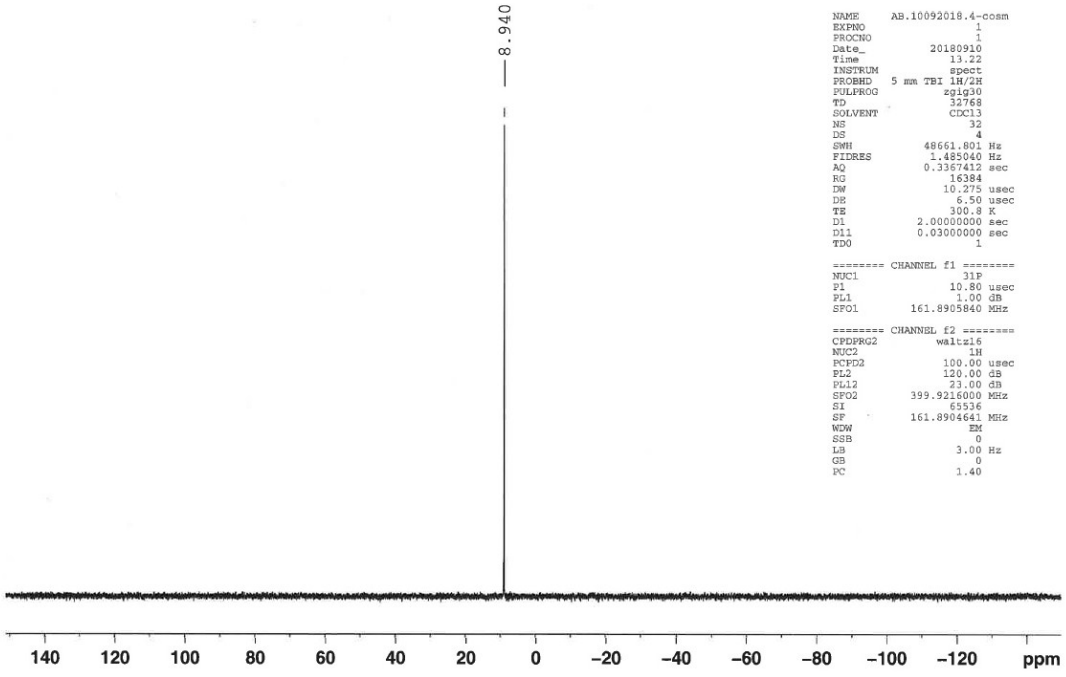


Figure SI1-10 : $^{31}P\{^{1}H\}$ NMR ($CDCl_3$) of compound **4d**

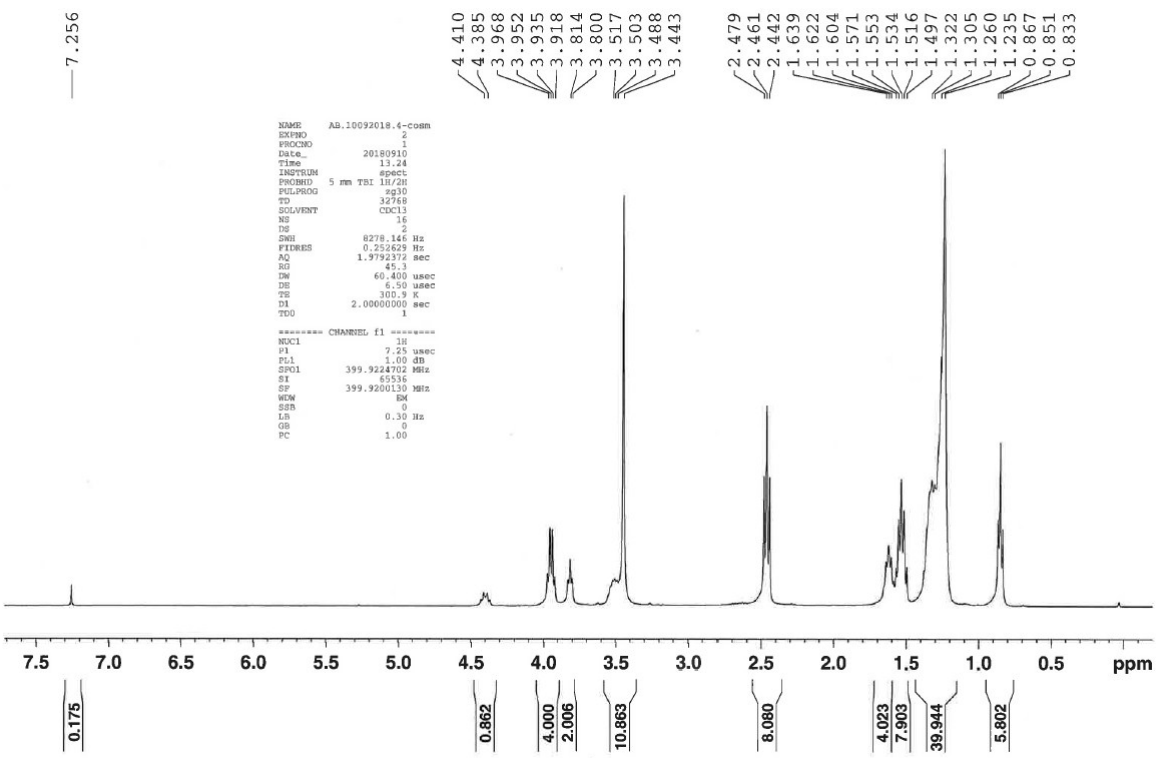


Figure SI1-11 : ^{1}H NMR ($CDCl_3$) for compound **4d**

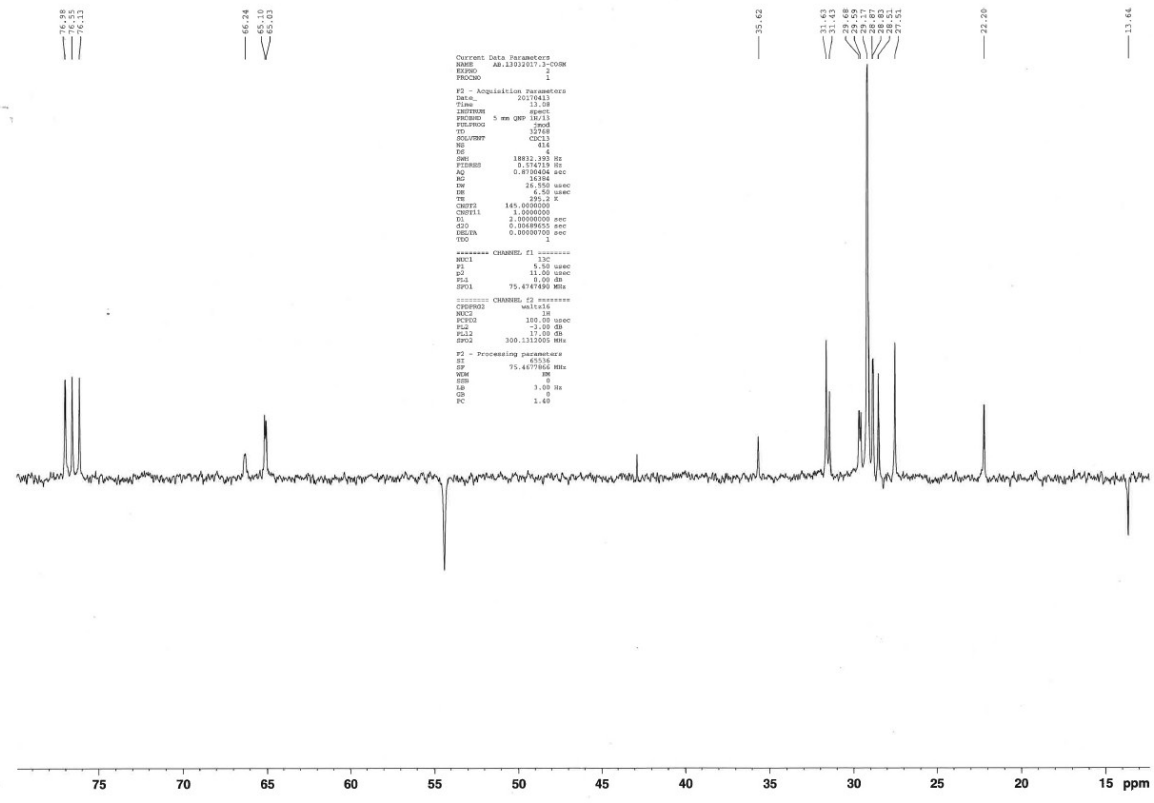


Figure SI1-12 : $^{13}C\{^{1}H\}$ NMR (Jmod; $CDCl_3$) for compound **4d**

2. Determination of CAC

Fluorescence was recorded on Agilent Cary Eclipse Fluorescence Spectrophotometer, using automatic polarizers for anisotropy measurements. The fluorescent probe 1,6-diphenylhaxatriene (DPH) was used for anisotropy measurements whereas the solvatochromic fluorescent probe Nile red was used for CMC determination. Both were purchased from Sigma-Aldrich.

Stock solutions of each lipid and each fluorescent probe were prepared in chloroform/methanol. For each sample, one lipid and one probe were then mixed in a glass tube and solvent was removed under reduced pressure. After solvent removal, pure water was added and let at 4°C for 3 nights. Solutions were then introduced in a bath at 60°C, vortexed and then sonicated during 20 min. For anisotropy measurements, the lipids concentration in the final preparation was $2x10^{-4}$ mol.L^{-1} whereas DPH concentration was $1x10^{-6}$ mol.L^{-1}.

About twenty samples of each lipid were prepared whose final concentration increases from $1x10^{-6}$ to $1x10^{-4}$ mol.L^{-1} whereas the Nile red concentration was always $3x10^{-7}$ mol.L^{-1}. Nile red was excited at 552 nm and fluorescence intensity was recorded at the maximum. The fluorescence intensity at maximum was plotted *versus* the log of the concentration (Figure 1). The CAC value can be seen as the onset of the fluorescence intensity increase (log C at the intersection of the lines). The equivalence point of the curve of the wavelength of the maximum versus C (*i.e* λ_{max} = f(C)) also reflects the CAC[1].

For all the compounds studied the values of the CAC were lower than $1.5x10^{-5}$ mol.L^{-1}.

[1] P.J.G. Coutinho, E.M.S. Castanheira, M. Céu Rei and M.E.C.D. Real Oliveira, *J. Phys. Chem. B,* 2002, **106**, 12841-12846

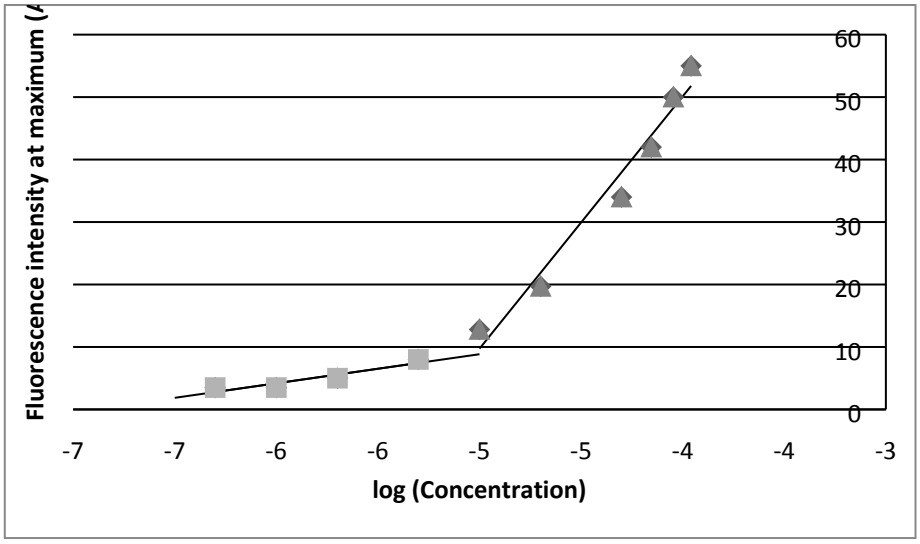


Figure SI2-1 : Fluorescence intensity *versus* log C for compound **4b**.

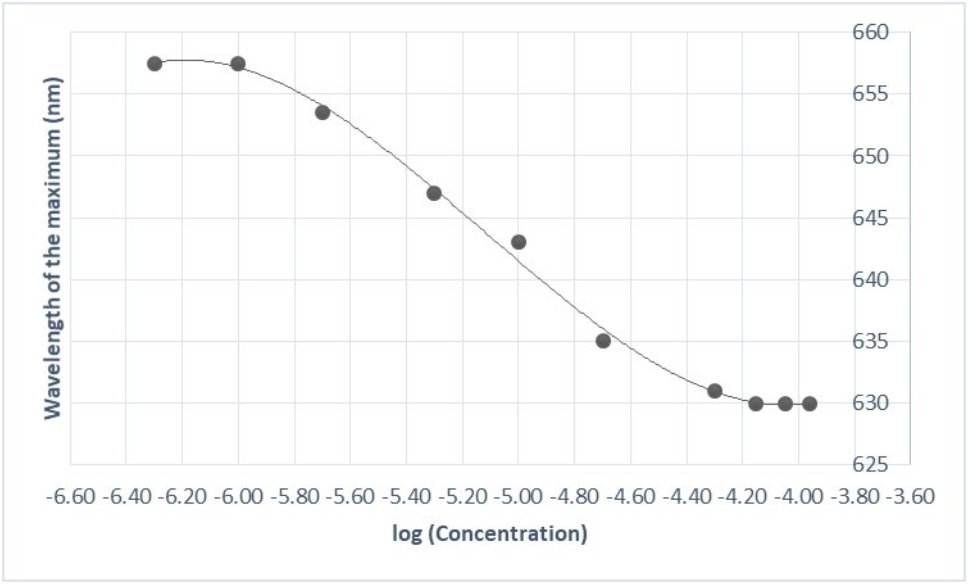


Figure SI2-2: λ_{max} *versus* log C for compound **4b**.

3. Formulations – DLS and zeta potential

An aliquot of a concentrated solution of the desired cationic lipid in chloroform was placed in a glass tube. Then the solvent was evaporated (4 h under vacuum) to get a thin lipid film to which 2 mL of sterile water was added and the film was hydrated for 18 h at 4°C. The solution was then allowed to go back to rt, vortexed for 30 s and sonicated for 30 min in an ultrasonic bath at 60°C. 100 µL of the liposomal solution was then diluted in 900 µL of sterile water then the size and the Zeta potential were measured.

	Size (nm)	PdI	Zeta (mV)
BSV36	198.3 ± 2.6	0.31	59.7 ±1.4
BSV101	200.3 ±2.4	0.35	48.7 ± 6.2
4a (BSV-S4)	75.3 ±5.9	0.35	58.7 ±3.1
4b (BSV-S6)	75.7 ±3.4	0.36	37.5 ±3.7
4c (BSV-S10)	89.7 ±1.7	0.24	55.9 ±1.0
4d (BSV-S12)	94.1 ±8.6	0.32	61.9 ± 3.4

Table SI3-1 : Size and Zeta potential of the prepared liposomal solutions

Size and Zeta potential measurements of the lipoplexes

20 µL of plasmid DNA (1 mg/mL) was placed in a 2 mL glass tube then 80 µL of sterile water was added. A volume V of the liposomal solution (1.5 mM) was added depending on the charge ratio (CR) and a volume V' of sterile water was added until having a total volume of 1 mL in each glass tube. The mixture was allowed to rest at rt for at least 45 min to ensure the DNA compaction.

CR	0.5	2	4	6
Volume V (µL)	20	80	160	320
Volume V'(µL)	880	820	740	480

Table SI3-2: Volumes of liposomal solution and sterile water added to prepare the lipoplexes.

	Charge Ratio (CR)			
	0.5	2	4	6
BSV36	123.1 ± 0.7	113 ± 4.5	59.1 ± 0.7	60 ± 0.5
BSV101	441.5 ± 7.2	195.3 ± 6.7	254.3 ± 3.8	118.9 ± 3.3
4a (BSV-S4)	1210 ± 14.8	226 ± 4.8	119.9 ± 2.6	181.4 ± 1.3
4b (BSV-S6)	172.6 ± 4.3	139.1 ± 5.3	68.9 ± 1.6	64.2 ± 0.4
4c (BSV-S10)	4845 ± 16.8	203.6 ± 11.4	191 ± 1.2	108.4 ± 0.4
4d (BSV-S12)	3880 ± 18.6	192.4 ± 4.8	163.8 ± 4.7	201.5 ± 1.7

Table SI3-3 : Size (nm) of lipoplexes prepared at different Charge Ratios (CR)

	Charge Ratio (CR)			
	0.5	2	4	6
BSV 36	8 ±1.0	47.4 ± 5.7	47.2 ± 1.4	49.4 ± 0.9
BSV 101	-18 ± 0.7	47.5 ± 2.5	44.7 ± 3.3	49.5 ± 2.8
4a (BSV-S4)	-2.8 ± 2.8	52.9 ± 2.1	51.7 ± 2.2	43.5 ± 2.3
4b (BSV-S6)	-27.6 ± 1.1	41.4 ± 0.4	61.7 ± 2.8	61.6 ± 3.4
4c (BSV-S10)	20.1 ± 2.5	52.4 ± 2.3	47.1 ± 2..9	58.6 ± 2.1
4d (BSV-S12)	15 ± 3.8	50.7 ± 1.8	52.9 ± 2.8	48.9 ± 3.1

Table SI3-4 : Zeta measurements (mV) of lipoplexes prepared at different Charge Ratios (CR)

4. 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 base line in the (π–A) isotherm indicated the cleanliness of the interface. The cationic lipid in $CHCl_3$/MeOH (2/1) mixture (25 μL) was slowly spread at the air-water interface using a Hamilton microliter syringe. After evaporation of the solvents, the isotherm of the lipid monolayer was measured by compressing the barriers at the rate of 5 cm^2/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 Delta (Δ, °) were reported in the **Table SI4-1**. Δ_{min} correspond to the value obtained at the lift off of the molecule (when the surface pressure began to increase) while Δ_{max} correspond to the collapse point.

	Delta min (n=2 or 3)	**Delta max (n=2 or 3)**	**Delta**
4a (BSV-S4)	3.9 ±0.1	8.6 ±0.1	4.7
4b (BSV-S6)	1.6 ±0.2	6.6 ±0.1	5
4c (BSV-S10)	1.4 ±0.2	6.8 ±0.2	5.4
4d (BSV-S12)	1.5 ±0.1	7.7 ±0.1	6.2
BSV 36	3.5 ±0.1	5.8 ±0.1	2.3
BSV 101	6.1 ±0.1	9.3 ±0.1	3.2

Table SI4-1: Values of the ellipsometric angle obtained at the beginning of the isotherm and at the collapse.

5. Molecular modeling

The alcohol **A**-**E** (**Figure SI5-1**) were used as models to determine the geometric features of lipid chains containing one sulfur atom at different position (molecules **A**-**D**). The geometry of compound **E** (C18- saturated alkyl chain) was also optimized as a reference compound. The geometry of the molecules **A-E** were optimized (gas phase) by using the DFT B3LYP method and employing 6-311+G** basis set for the molecules **A**-**D** and 6-31+G* for compound **E**. The geometry optimization was achieved with Spartan '16, Version 2.0.7, Aug 1 2017. Selected angles and bond lengths were reported in **Table SI5-2** for compounds **A**-**D** and in **Table SI5-3** for compound **E**. The bend induced by the presence of the sulfur atom was estimated by measuring for each compound three angles close to the sulfur atom for compounds **A**-**D** (see **Table SI5-4** for details). The bend of the saturated lipid chain (compound **E**) was estimated by measuring angles involving the atoms C1 to C8 for this compound **E** (see **Table SI5-5** for details). Ball and stick representations of compounds **A**-**E** are depicted in **Table SI5-6**

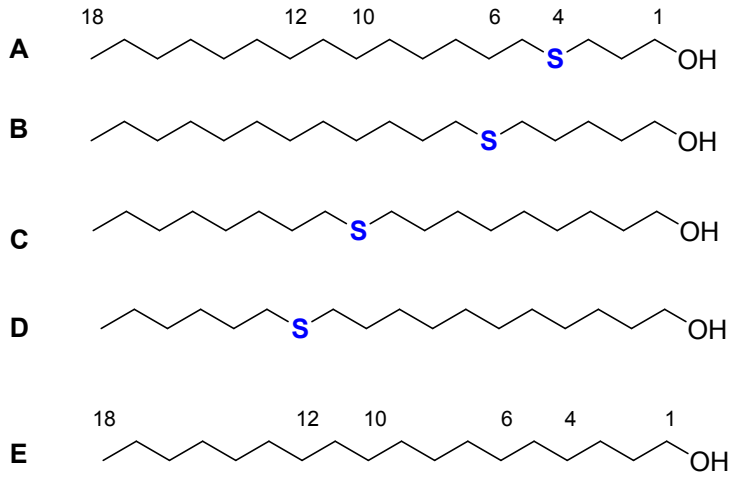


Figure SI5-1: Chemical structure and labelling of the model used for this computational study

	Angle C-S-C (°)	Distances (Å)	Distances (Å)
A	99.92	S-C5: 1.838	C5-C6: 1.529
B	99.99	S-C7 : 1.838	C7-C8: 1.529
C	100.19	S-C11: 1.838	C11-C12: 1.529
D	100.33	S-C12: 1.838	C13-C14: 1.529

Table SI5-2: Selected angles and distances for compounds **A-D**

	Angle C3-C4-C5 (°)	Distances (Å)
E	113.52	C3-C4: 1.534 C4-C5: 1.534

Table SI5-3: Selected angles and distances for compounds **E**

	Angle (°)	Average angle (°)
A	C1-C3-C5: 175.51 C2-C6-C8: 175.38 C3-C5-C7: 175.63	175.5
B	C3-C5-C7: 175.88 C4-C8-C10: 175.53 C5-C7-C9: 175.72	175.7
C	C7-C9-C11: 175.66 C8-C12-C14: 175.11 C9-C11-C13: 175.91	175.6
D	C9-C11-C13: 175.35 C10-C14-C16: 175.19 C2-C14-C18: 176.31	175.6

Table SI5-4: Selected angles and average value to estimate the bend induced by the presence of a thioether in compounds **A-D**

	Angle (°)	Average angle(°)
E	C1-C3-C5: 179.39 C2-C4-C6: 179,84 C3-C5-C7: 179.09 C4-C6-C8: 179.74	179.5

Table SI5-5: Selected angles and average value to estimate the bend of the saturated lipid chain present in compound **E**.

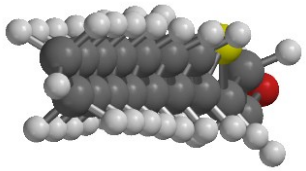
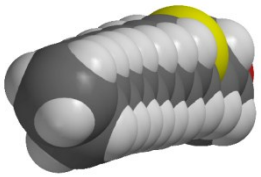
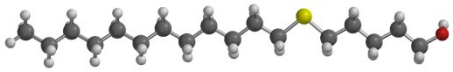
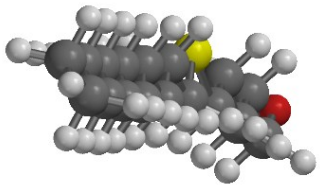
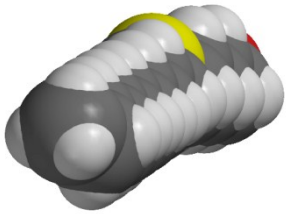
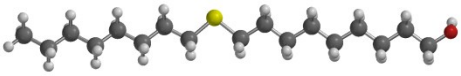
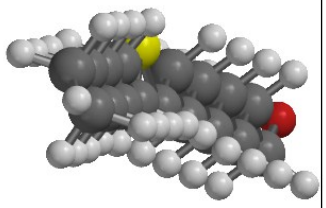
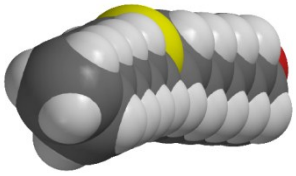
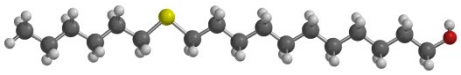
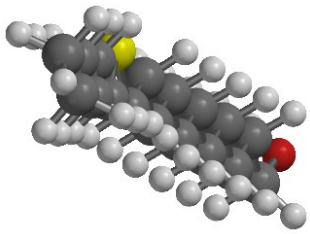
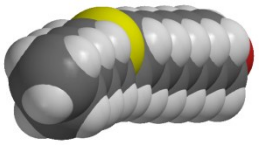
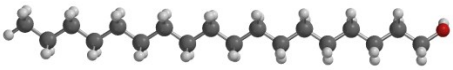
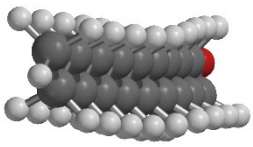
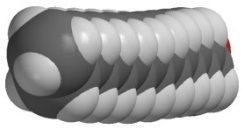
Label	Ball and stick	Ball and stick	Compact model
A			
B			
C			
D			
E			

Table SI5-6: ball and stick and compact models for compounds **A-E** (sulfur atom in yellow; oxygen atom in red, carbon atoms in black and hydrogen atoms in grey).

6. ^{31}P Solid state NMR

Preparation of the sample: 200 mg of compound (**4d)** was dissolved in 1 mL of chloroform and then placed in a glass tube. The solvent was then slowly evaporated to give a lipid film. 1 mL of sterilized water was added and the mixture was sonicated for 10 min till the dispersion of the film. The obtained mixture was then lyophilized during the night to give a fluffy powder to which 1 mL of sterilized water was added and the sample was placed in liquid nitrogen for 10 min, warmed to room temperature then placed in a water bath at 50°C for 20 min and finally vortexed for 15 s. This freeze thaw shaking cycle was repeated three times to obtain a gel which was placed inside a rotor and in an NMR tube.

^{31}P NMR (**Figure SI6-1**): the spectrum was acquired by using Hahn echo sequence (90°-τ-180°-τ-acq.). The acquisition spectral window was 200 KHz, the $\pi/2$ pulse width was 4.88µs, the recycling delay was 5s and echo delay of 40µs. The number of acquisitions was 10000 scans.

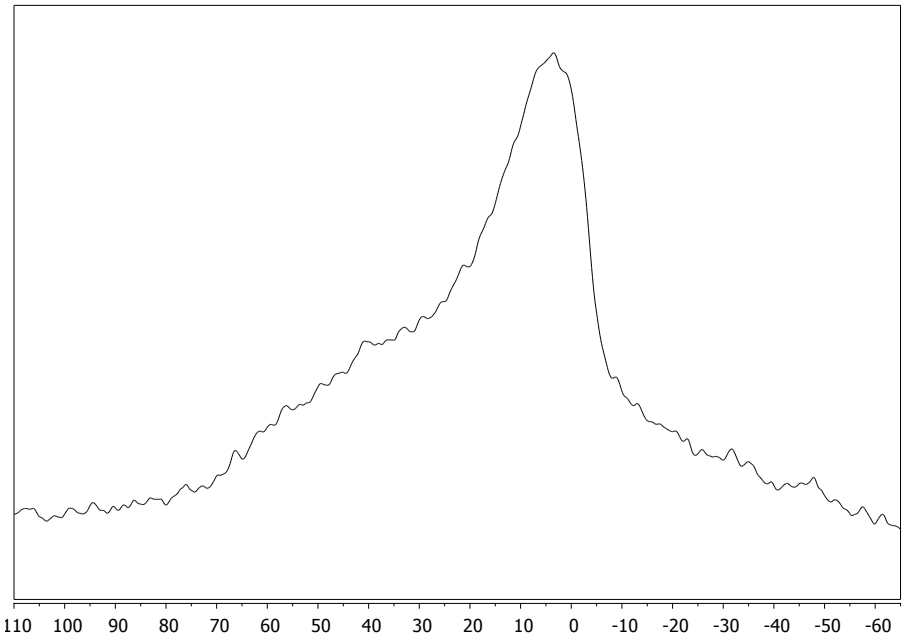


Figure SI6-1: ^{31}P Solid state NMR (Hahn echo sequence) spectra for compound **4d**

7. Transfection efficacy

Transfection with 0.25 µg pGM144 / well

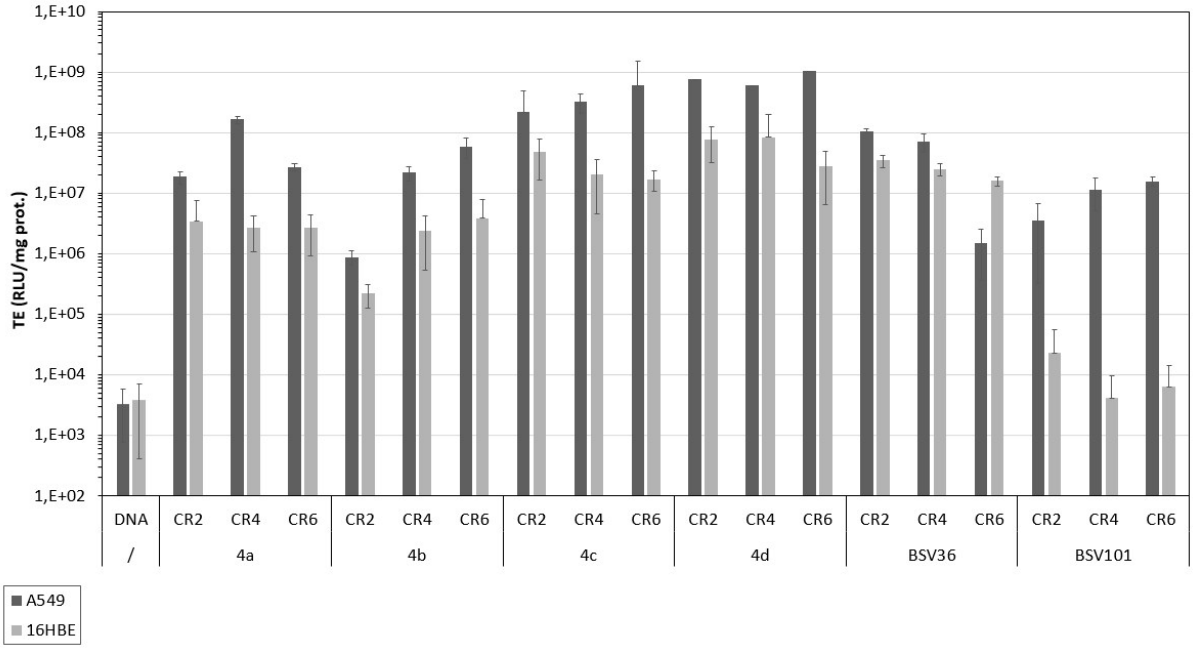


Figure SI7-1: In vitro Transfection Efficacy (TE) on A549 and 16HBE cell lines at different charge ratio (CR = 2, 4 and 6) with pGM144 plasmid encoding luciferase used at 0.25µg/well. Uncomplexed pDNA was used as negative controls. Results are expressed as the Relative Light Units/mg of total proteins obtained with each formulation, reflecting their transfection abilities, as the mean ± SD of 3 wells.

Transfection of A549 cell line with 0.1µg pGM144 /well

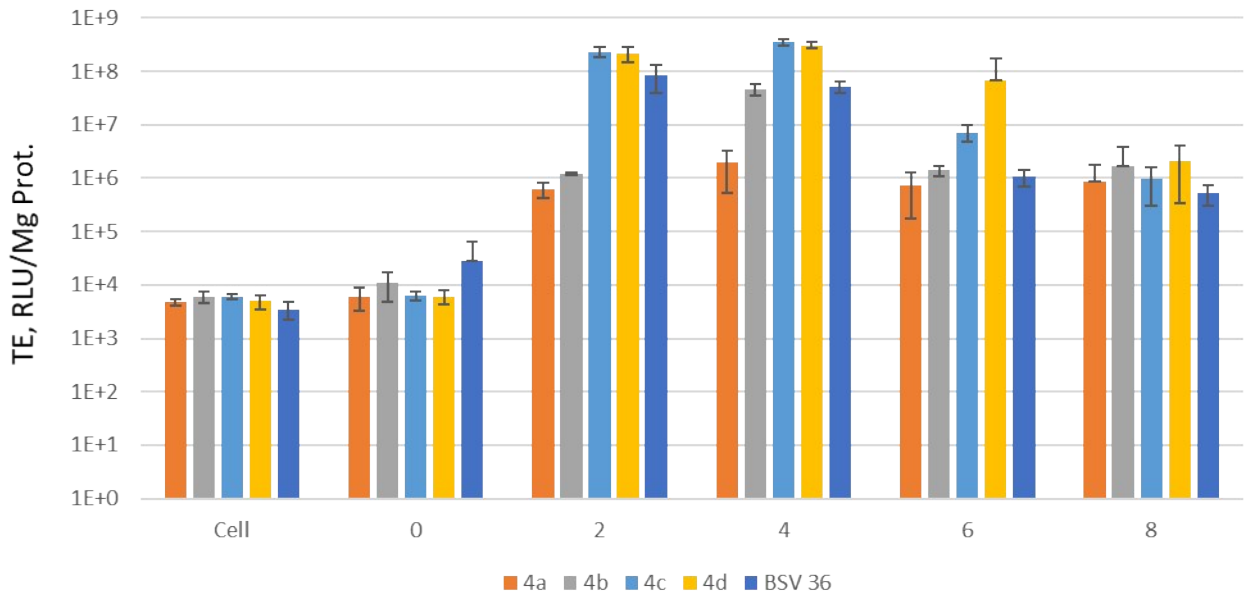


Figure SI7-2: In vitro Transfection Efficacies of compounds **4a-d** and **BSV36** on A549 cell line with 0.1 µg of pGM144/well at different charge ratio (2, 4, 6 and 8). Uncomplexed pDNA (CR = 0) and untransfected cells were used as negative controls. Results are expressed as the Relative Light Units/mg of total proteins obtained with each formulation, reflecting their transfection abilities, as the mean ± SD of 3 wells.

8. Viability

8-1 Viability by bioluminescence quantification of cellular ATP (ViaLight kit) – 16HBE cell line

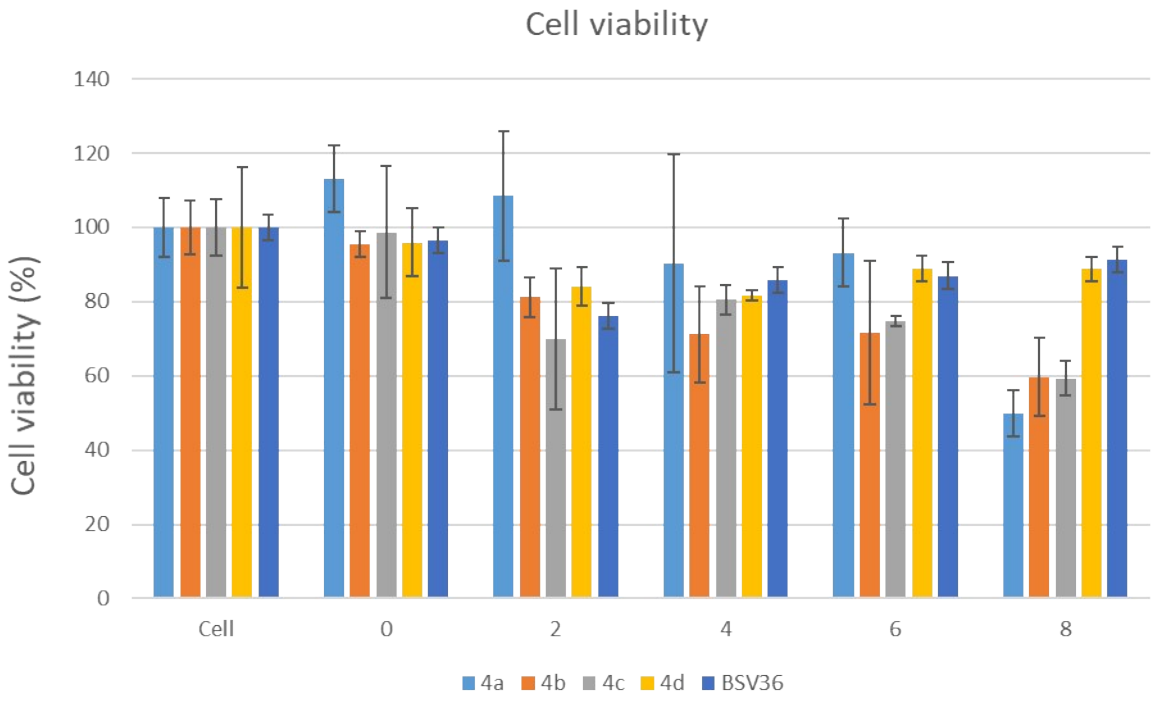


Figure SI8-1: Cell viability of 16HBE cell line determined at charge ratio (CR = 2, 4, 6 and 8) for compounds **4a-d** and **BSV36** using the vialight kit (Lonza). It was used to measure the ATP content of the cells which was indicative of cell viability and proliferation. Cell viability was determined 24 h after deposition. In every test, the amount of DNA (0.25µg) introduced per well was the same. The viability was calculated considering untreated cells as reference (100%) (mean+/- SD with n=3) and CR=0 corresponds to the unprotected pDNA.

8-2 MTT assays for A549 cells

For A549 cell line we had some difficulties with the vialight kit (Lonza) to determine the toxicity (unexpected high value of viability was observed at high CR whereas the maximum of toxicity was mesured at CR=2 or 4). In consequence, we decided to determine the toxicity by MTT assays.

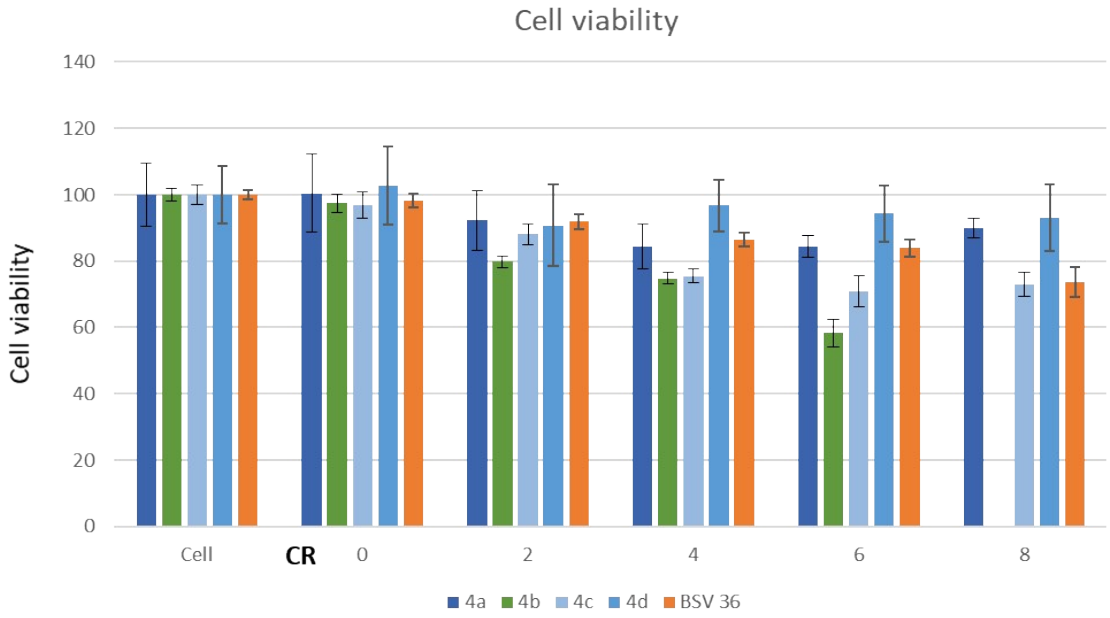


Figure SI8-2: Cell viability of A549 cell line determined by MTT assays at different Charge Ratio (CR = 2, 4, 6 and 8) for compounds **4a-d** and **BSV36**. After 24 h transfection, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, 5 mg/mL in PBS) was added to each well at a final concentration of 0.5 mg/mL per well, and the cells were incubated for 4 h at 37°C. MTT converted in formazan was solubilized with 10% sodium dodecyl sulfate diluted in hydrochloric acid and incubated overnight at 37°C. The absorbance was measured at 600 nm with spectrophotometer and expressed as a percentage of untransfected cells. In every test, the amount of DNA (0.25µg) introduced per well was the same. The viability was calculated considering untreated cells as reference (100%) (mean+/- SD with n=3) and CR=0 corresponds to the unprotected pDNA.