Electronic Supplementary Material (ESI) for Nanoscale.

Supporting Information

Cationic Switchable Lipids: pH-Triggered Molecular Switch for siRNA Delivery

Warren Viricel[1], *Steve Poirier*[2], *Amira Mbarek*[1], *Rabeb Mouna Derbali*[1], *Gaetan Mayer*[1,2], *Jeanne Leblond*[1]*

[1] Faculty of Pharmacy, University of Montreal, P.O. Box 6128, Downtown Station, Montreal, QC, Canada

[2] Laboratory of Molecular Cell Biology, Montreal Heart Institute, Montreal, H1T 1C8, QC, Canada

*Address correspondence to jeanne.leblond-chain@umontreal.ca

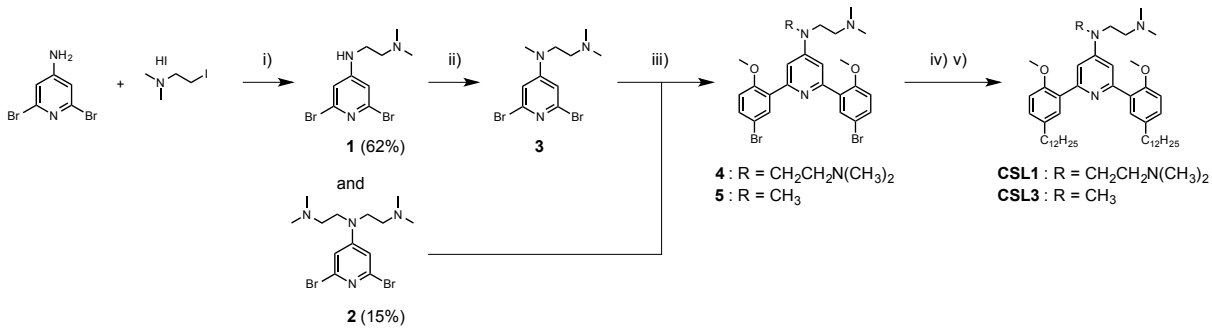
Table of figures

Table of content

Abbreviations

AcOEt: ethyl acetate; $C_{12}E_8$: octaethylene glycol monododecyl ether; CSL: cationic switchable lipid; DCM: dichloromethane; DIPA: diisopropylamine; DMEM: Dulbecco's Modified Eagle's Medium, DMF: dimethylformamide; DMG-PEG_{2000}: 1,2-dimyristoyl-sn-glycerol methoxypolyethyleneglycol 2000; DMSO: dimethyl sulfoxide; DSPC: (1,2-distearoyl-sn-glycero-3-phosphocholine; DSPE-PEG_{2000}: N-(carbonyl-methoxypolyethyleneglycol 2000)-1,2-distearoyl-sn-glycero-3-phosphoethanolamine; EDTA: ethylenediaminetetraacetic acid; EIPA: 5-(N-Ethyl-N-isopropyl)amiloride; EMEM: Eagle's Minimum Essential Medium; EtOH: ethanol; FBS : fetal bovine serum; FVII: coagulation factor VII; GFP: green fluorescent protein; HEPES: 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; LDLR: low density lipoprotein receptor; LNP: lipid nanoparticle; MeOH : methanol; PBS : phosphate buffer saline; PCSK9: proprotein convertase subtilisin/kexin type 9; PEG: poly(ethyleneglycol); POPC: 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine; R18: octadecyl rhodamine B; TBAF: tetra-n-butylammonium fluoride; TEAA: triethylammonium acetate; TFA: trifluoroacetic acid; THF: tetrahydrofuran.

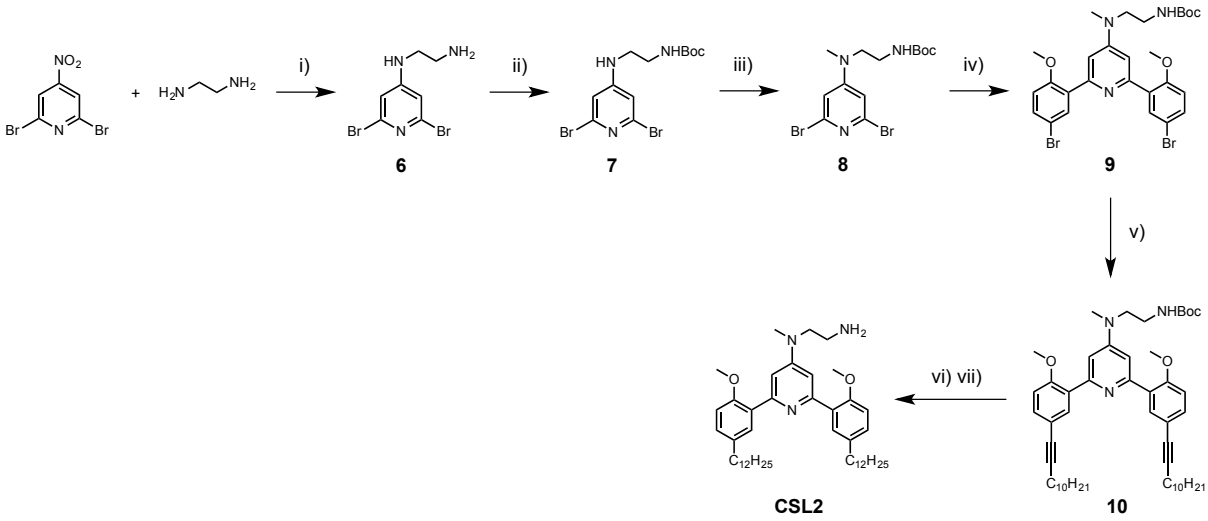
Scheme S1. Synthesis pathway of the cationic switchable lipids CSL1 and CSL3



i) NaH, THF, 80°C, 0.5h ; ii) ICH_3, NaH, THF, 40°C, 2h ; iii) 5-bromo-2-methoxyphenylboronic acid, $Pd(PPh_3)_4$, Na_2CO_3, THF/H_2O, 80°C, 48h ; iv) 1-dodecyne, $PdCl_2(PPh_3)_2$, PPh_3, TBAF, DMF/DIPA, 80°C, 16h ; v) H_2, Pd/C, EtOH, room temperature, 4h.

The synthesis and characterization of the compounds are further detailed below.

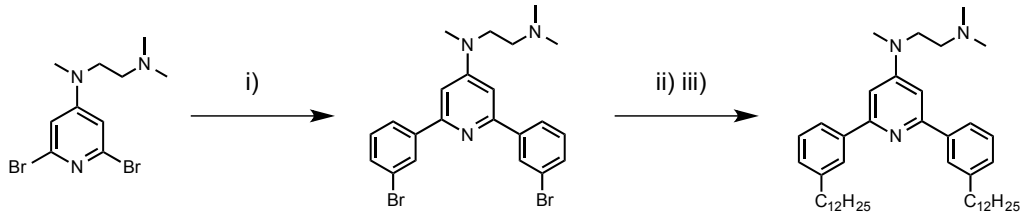
Scheme S2. Synthesis pathway of the cationic switchable lipid CSL2



i) NaH, DMF, room temperature, 3h ; ii) Boc_2O, DCM, room temperature, 2h ; iii) ICH_3, NaH, DMF, 40°C, 3h ; iv) 5-bromo-2-methoxyphenylboronic acid, $Pd(PPh_3)_4$, Na_2CO_3, THF/H_2O, 80°C, 48h ; v) 1-dodecyne, $PdCl_2(PPh_3)_2$, PPh_3, TBAF, DMF/DIPA, 80°C, 16h ; vi) H_2, Pd/C, EtOH, room temperature, 16h ; vii) HCl 4M, dioxane, room temperature, 2h.

The synthesis and characterization of the compounds are further detailed below.

Scheme S3. Synthesis pathway of the negative control cationic lipid CSL4

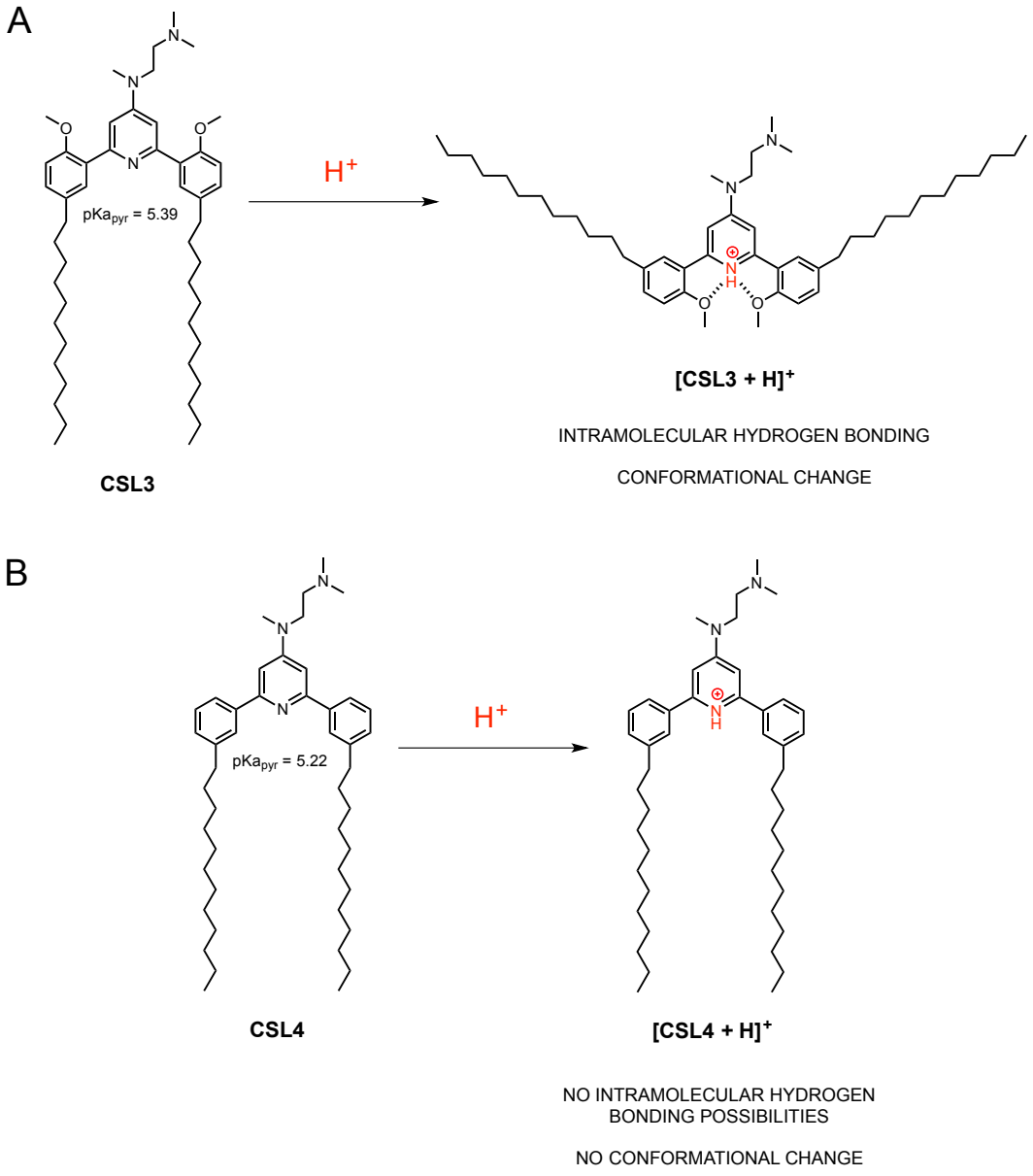


3 **11** **CSL4**

i) 3-bromophenylboronic acid, $Pd(PPh_3)_4$, Na_2CO_3, THF/H_2O, 80°C, 24h ; ii) 1-dodecyne, $PdCl_2(PPh_3)_2$, PPh_3, TBAF, DMF/DIPA, 80°C, 16h ; iii) H_2, Pd/C, EtOH, room temperature, 4h.

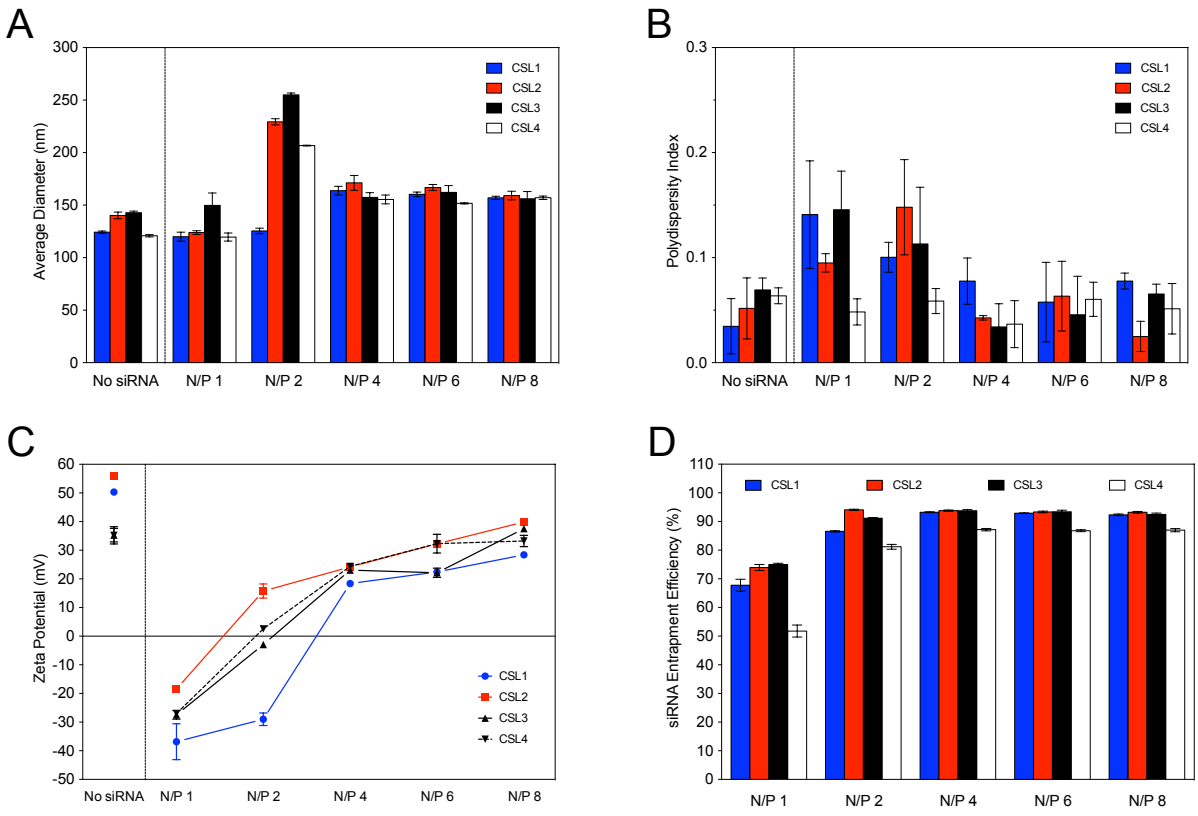
The synthesis and characterization of the compounds are further detailed below.

Scheme S4. Protonation induced conformational change of the cationic switchable lipids CSL3 and CSL4 (negative control lipid)



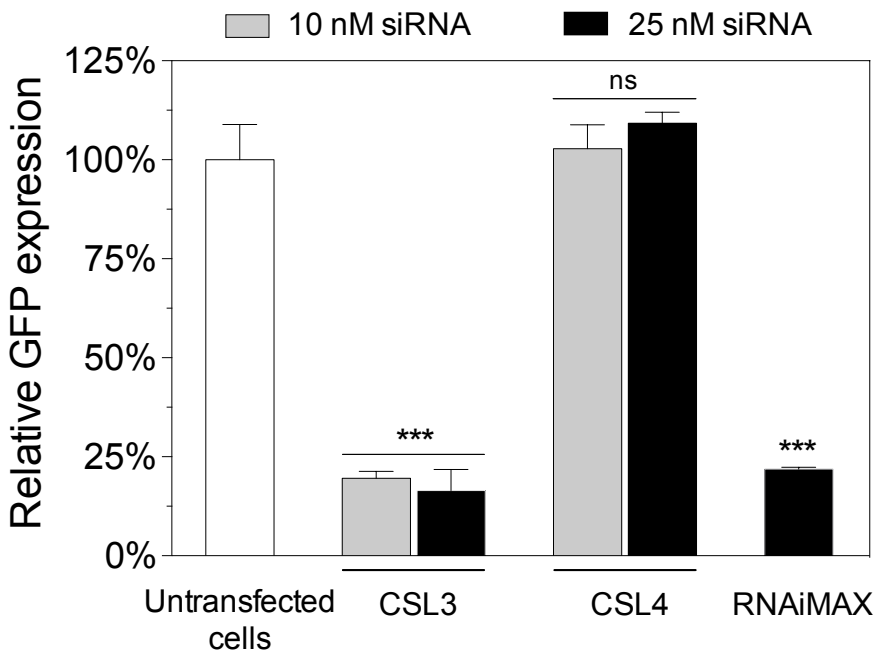
(A) Upon protonation of the central pyridine ring in the cationic switchable lipid CSL3, the formation of intramolecular hydrogen bonding between the protonated nitrogen and the two methoxy moieties leads the conformation to freeze, destabilizing the lipid nanoparticle and provoking endosomal escape. This behavior was observed in a previous work.[1] **(B)** The negative control cationic lipid CSL4 lacks the two methoxy moieties required to perform intramolecular hydrogen bonding with the protonated pyridine, and is therefore unable to freeze its conformation at endosomal pH values. Note that the pKa$_{pyr}$ values of the central pyridine ring of these two lipids are similar (5.39 and 5.22), as predicted *in silico* (CSpKa^{TM} software, ChemSilico LLC.).

Figure S5. Physicochemical characterisation of LNP/siRNA complexes made by manual extrusion



Physicochemical characterisation of the lipid nanoparticles encapsulating anti-GFP siRNA at different lipid nitrogen/siRNA phosphate (N/P) ratios. Lipid nanoparticles were prepared using cationic switchable lipids, DSPC, cholesterol and DSPE-PEG_{2000} at a molar ratio of 50:10:37.5:2.5 respectively. Size **(A)** and polydispersity index **(B)** of the lipid nanoparticles/siRNA complexes were measured by dynamic light scattering in Opti-MEM®. Zeta potential **(C)** of lipid nanoparticles/siRNA complexes were measured in dextrose 5%. siRNA encapsulation efficiency **(D)** was measured using a SYBR® Gold fluorescence assay.

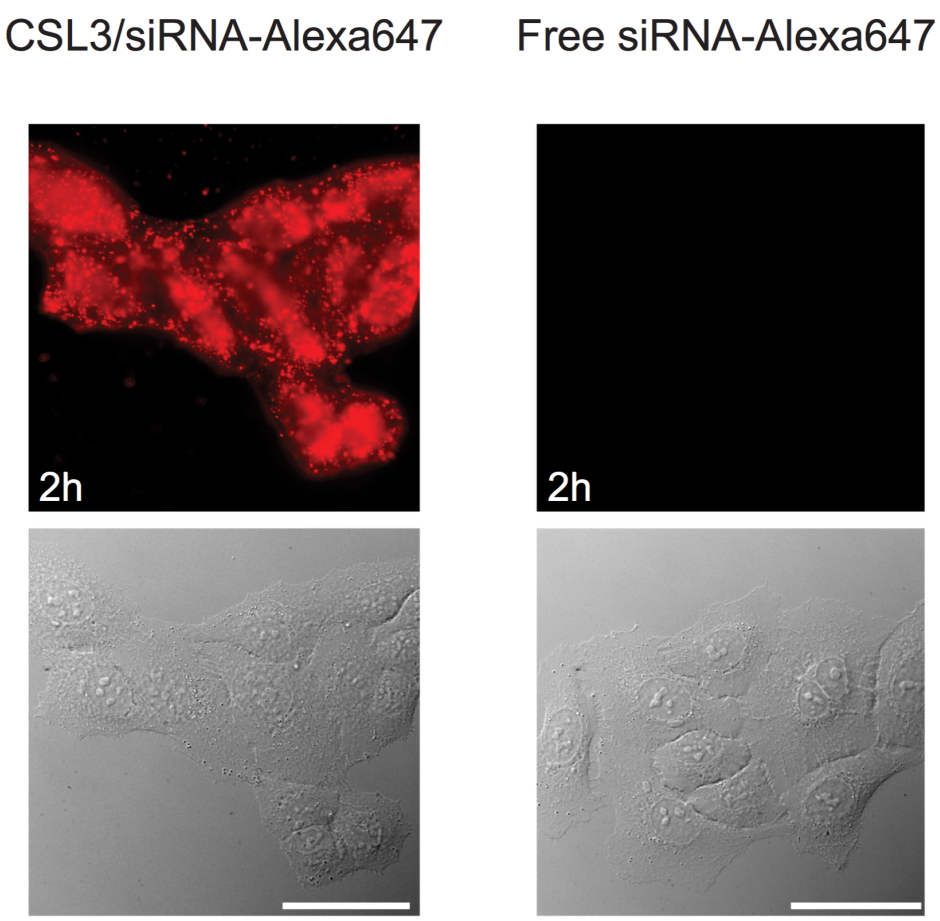
Figure S6. Forward transfection on HeLa/GFP cells with CSL3 and CSL4 LNP



Forward transfection on HeLa/GFP cells with CSL3 and CSL4-based LNP (CSL, DSPC, cholesterol and DSPE-PEG_{2000} at a molar ratio of 50:10:37.5:2.5 respectively – made by manual extrusion). Gene knockdown was assayed by flow cytometry after a 48h incubation period (n = 3). Statistical analysis performed with two-tailed Student's t-test: ns. $p>0.05$; ***$p<0.001$.

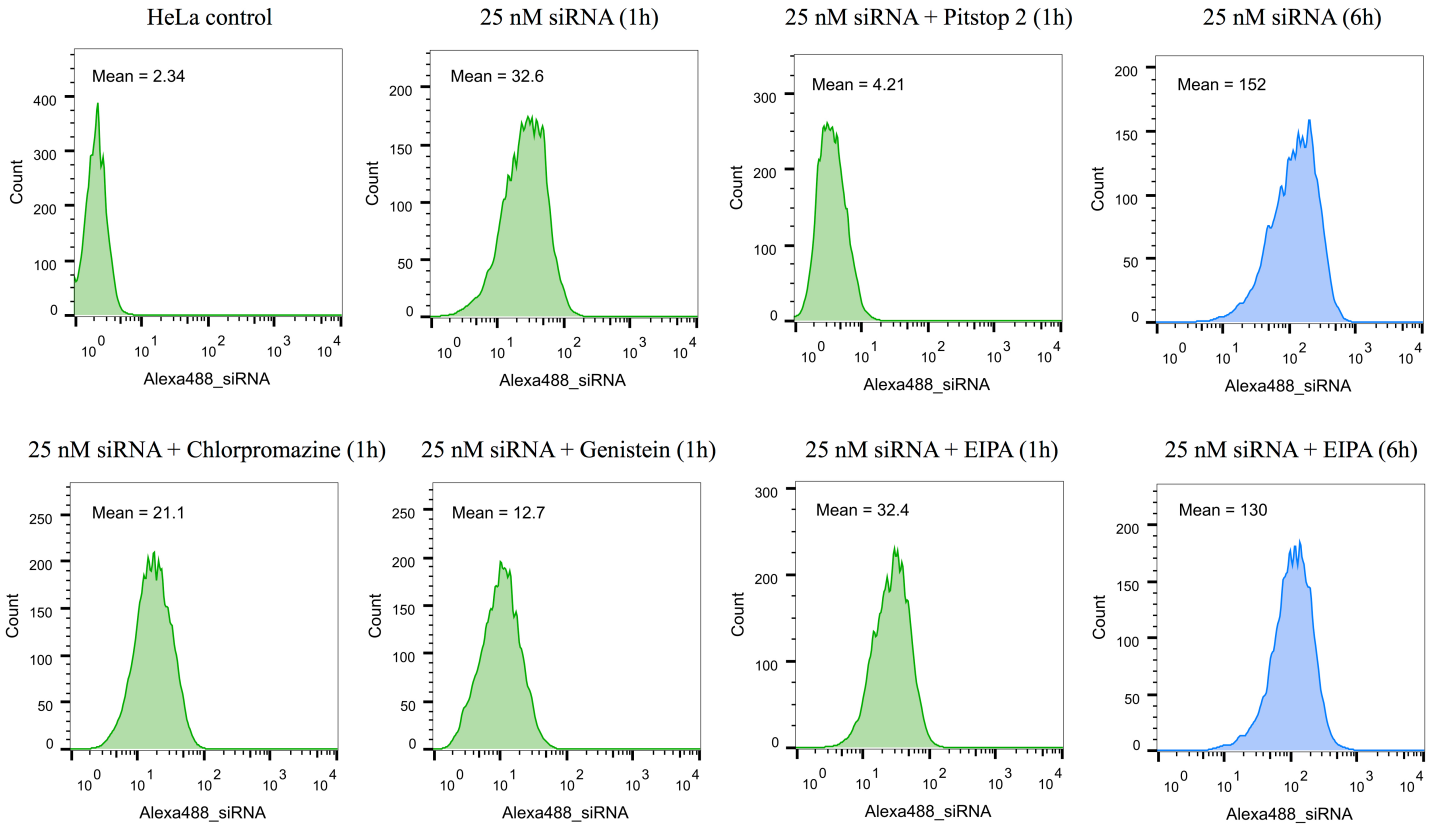
Forward transfection was realized to make sure that transfection efficiency of CSL3 and CSL4 LNP remains similar, regardless of the chosen transfection method (reverse or forward transfection).

Figure S7. CSL3/siRNA-Alexa647 and free siRNA-Alexa647 delivery on HeLa cells



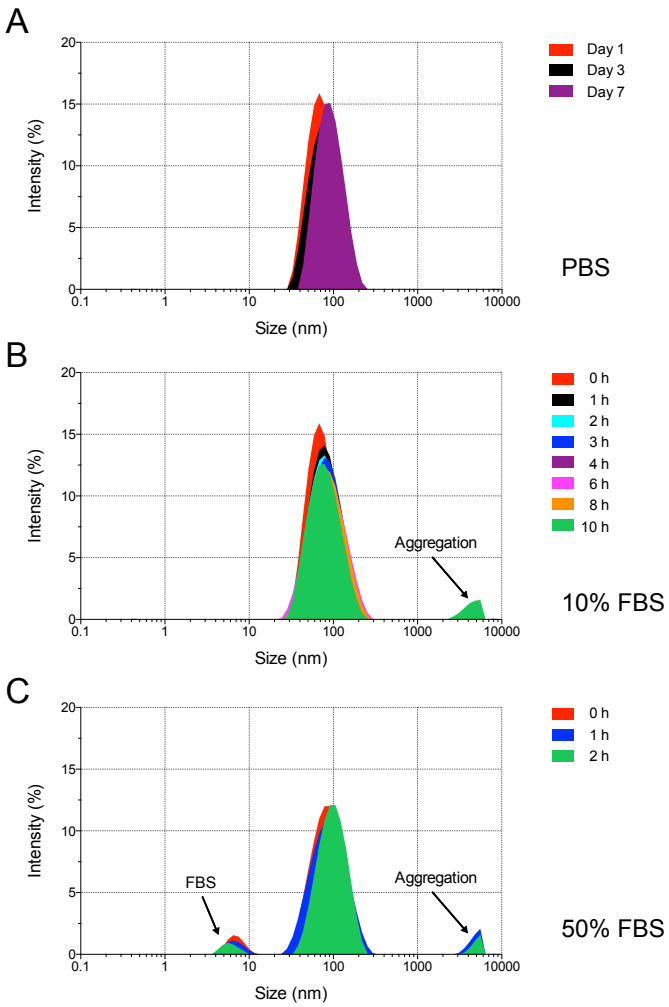
2-hour incubation of HeLa cells with CSL3/siRNA-Alexa647 LNP (CSL3, DSPC, cholesterol and DSPE-PEG_{2000} at a molar ratio of 50:10:37.5:2.5 respectively – made by manual extrusion) or free siRNA-Alexa647. Final siRNA-Alexa647 concentration in the dish is 50 nM. Representative pictures are shown. Scale bar is 20 µM.

Figure S8. Flow cytometry profiles of uptake studies with endocytosis inhibitors



Representative flow cytometry profiles of cell uptake (1-hour incubation (green) or 6-hour incubation (blue)) of the CSL3-based lipid nanoparticles formulation (CSL3/DSPC/cholesterol/DSPE-PEG_{2000} 50:10:37.5:2.5 mol% - made by manual extrusion) encapsulating siRNA-Alexa488 (25 nM/well), in the presence or absence of endocytosis inhibitors. HeLa cells were used for this study. Clathrin (Chlorpromazine – 10 µg/mL), caveolae (Genistein – 200 µM), clathrin/caveolae (Pitstop 2 – 20 µM) or macropinocytosis (EIPA – 50 µM) endocytic pathways were inhibited. Experiment was realised in triplicate.

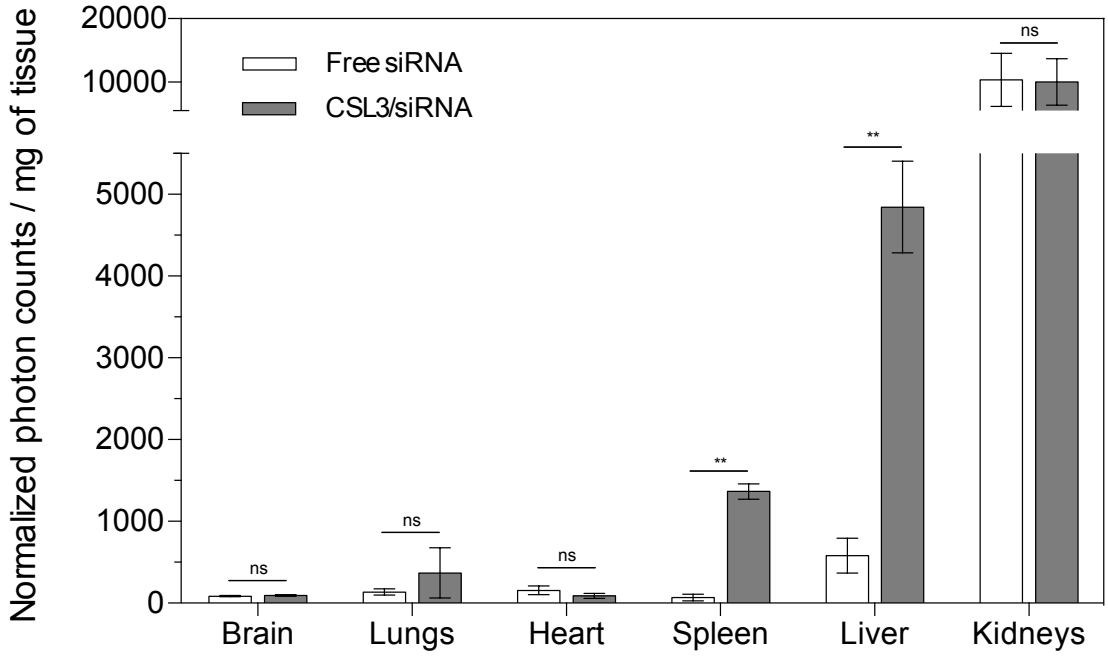
Figure S9. Stability study of CSL3 LNP made by microfluidic mixing



CSL3 LNP (CSL3/DSPC/cholesterol/DMG-PEG$_{2000}$ 50:10:37.5:2.5 mol% - made by microfluidic mixing) were incubated in PBS buffer containing 0% (A), 10% (B) or 50% (C) FBS.

Upon storage in PBS at +4°C, CSL3-based LNP are stable for >7 days. When incubated in buffer containing 10% FBS, LNP remain stable for 10h at 37°C. Nevertheless, aggregation occurs at the 10-hour and 1-hour time point in 10% and 50% FBS respectively. This partial serum instability could result in the premature release of siRNA in the blood stream following intravenous injection, explaining the relatively high fluorescent signal observed in the kidneys in the biodistribution study (see Figure S10).

Figure S10. Quantification of biodistribution of siRNA-Cy5 encapsulated into CSL3 LNP



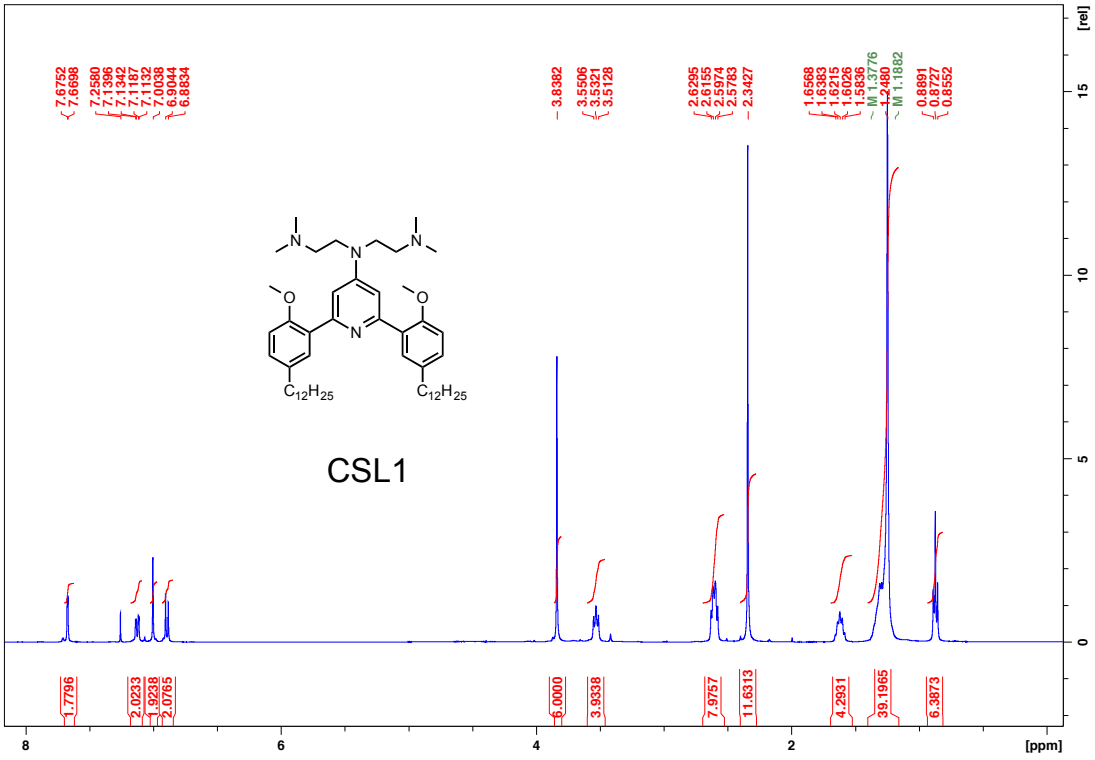
Quantification (photon counts normalized by organ wet mass) of siRNA-Cy5 biodistribution. siRNA-Cy5 were encapsulated into CSL3 LNP (CSL3/DSPC/cholesterol/DMG-PEG_{2000} 50:10:37.5:2.5 mol% - made by microfluidic mixing). Organs were harvested and imaged 4 hours after tail vein injection at a siRNA-Cy5 dose of 1.5 mg/kg. Experiment was realized in triplicate. Statistical analysis performed with two-tailed Student's t-test: ns. $p>0.05$; *$p<0.05$; **$p<0.01$.

Table S11. siRNA sequences used in this study

siRNA	Sense strand	Antisense strand	Modification	Origin
siRNA GFP	Not provided	Not provided	None	Dharmacon (cat# P-002048-01-20)
siRNA-Alexa647	Not provided	Not provided	3'-Alexa Fluor 647 on the sense strand	Qiagen AllStars Negative Control siRNA (cat# 1027287)
siRNA-Alexa488	Not provided	Not provided	3'-Alexa Fluor 488 on the sense strand	Qiagen AllStars Negative Control siRNA (cat# 1027284)
siRNA-Cy5	5'-Cy5-UAGCGACUAAACACAUCAAUU-3'	5'-UUGAUGUGUUUAGUCGCUAUU-3'	5'-Cy5 on the sense strand	Dharmacon (siGENOME Non-Targeting Control siRNA)
siRNA PCSK9	5'-GccuGGAGuuuAuucGGAAdT*dT-3'	5'-UUCCGAAuAAACUCcAGGCdT*dT-3'	None	Dharmacon
siRNA FVII	5'-GGAfUfCAfUfCfUfCAAGfUfCfUfUAfCdT*dT-3'	5'-GfUAAGAfCfUfUGAGAfUGAfUfCfCdT*dT-3'	None	Dharmacon

2'-OMe modified nucleotides are in lower case. 2'-F modified nucleotides are denoted by "f". Phosphorothioate linkages are represented by asterisks. siRNA PCSK9 and siRNA FVII sequences were obtained from the literature.[2,3]

Figure S12. ^{1}HNMR and ^{13}CNMR spectrums of compound CSL1



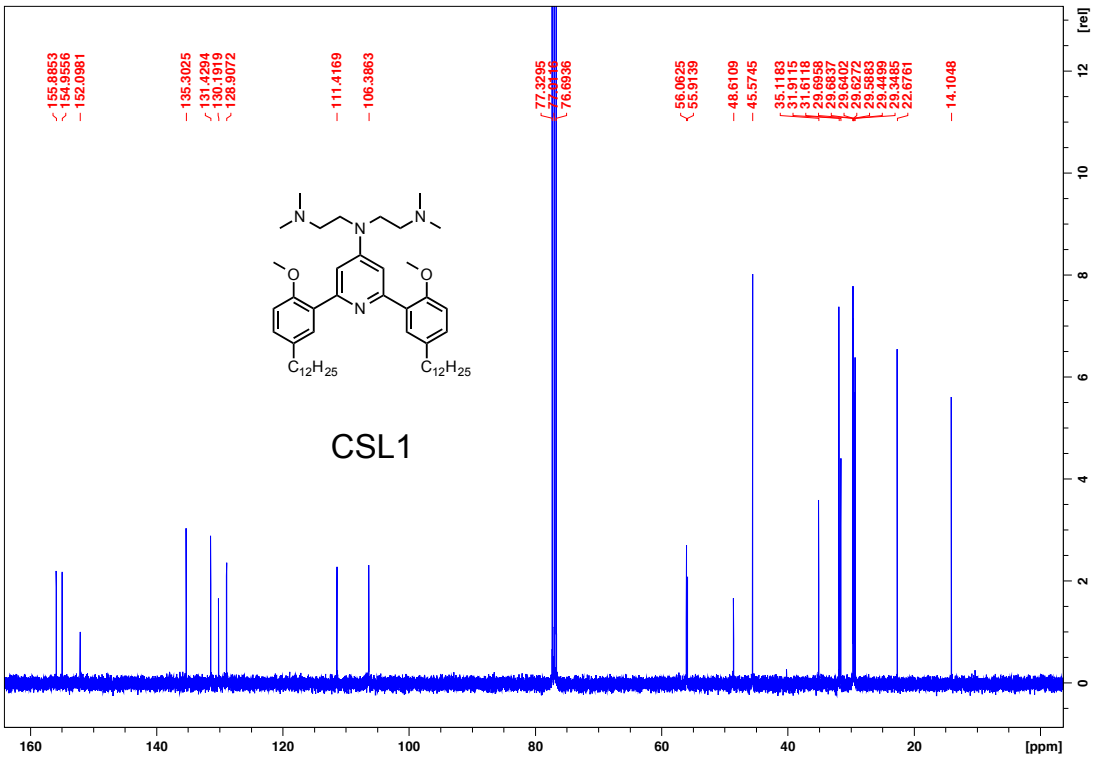
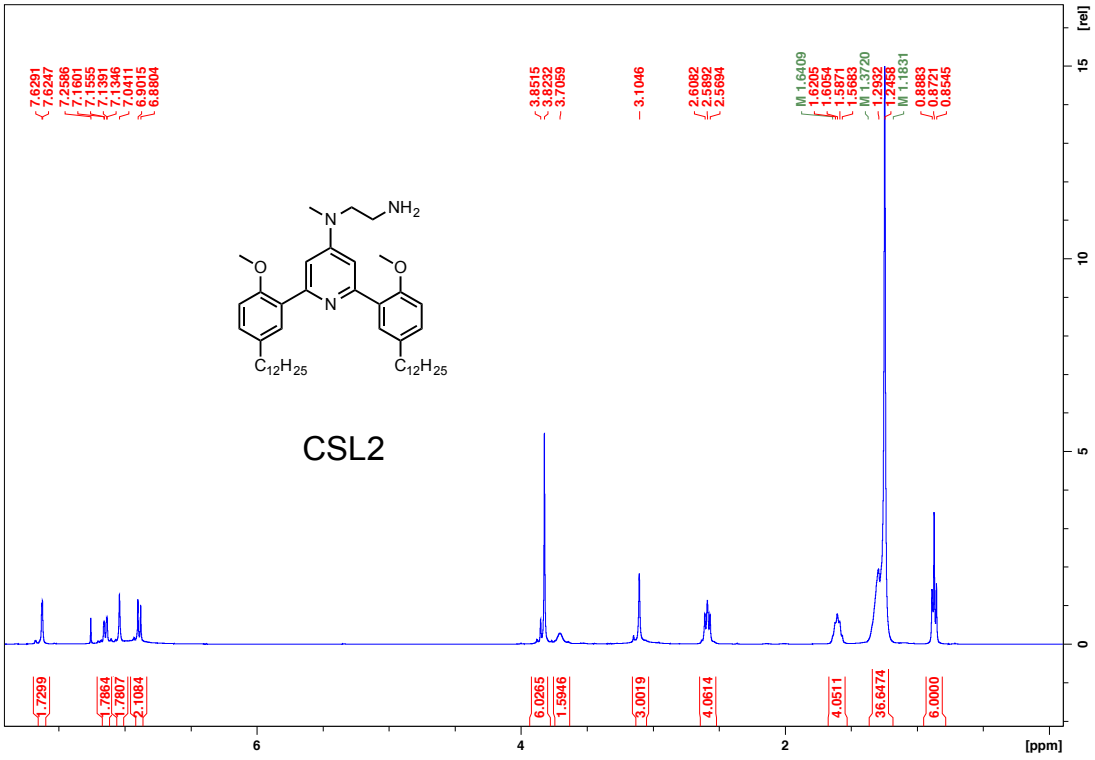


Figure S13. ^{1}HNMR and ^{13}CNMR spectrums of compound CSL2



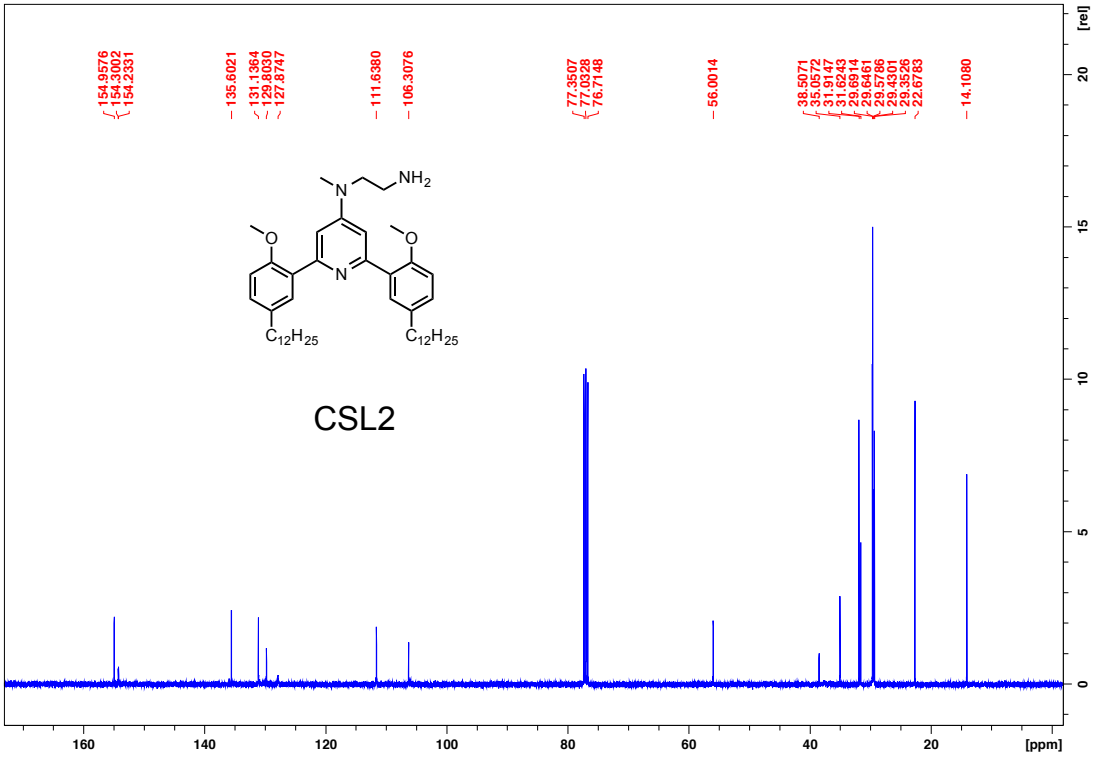
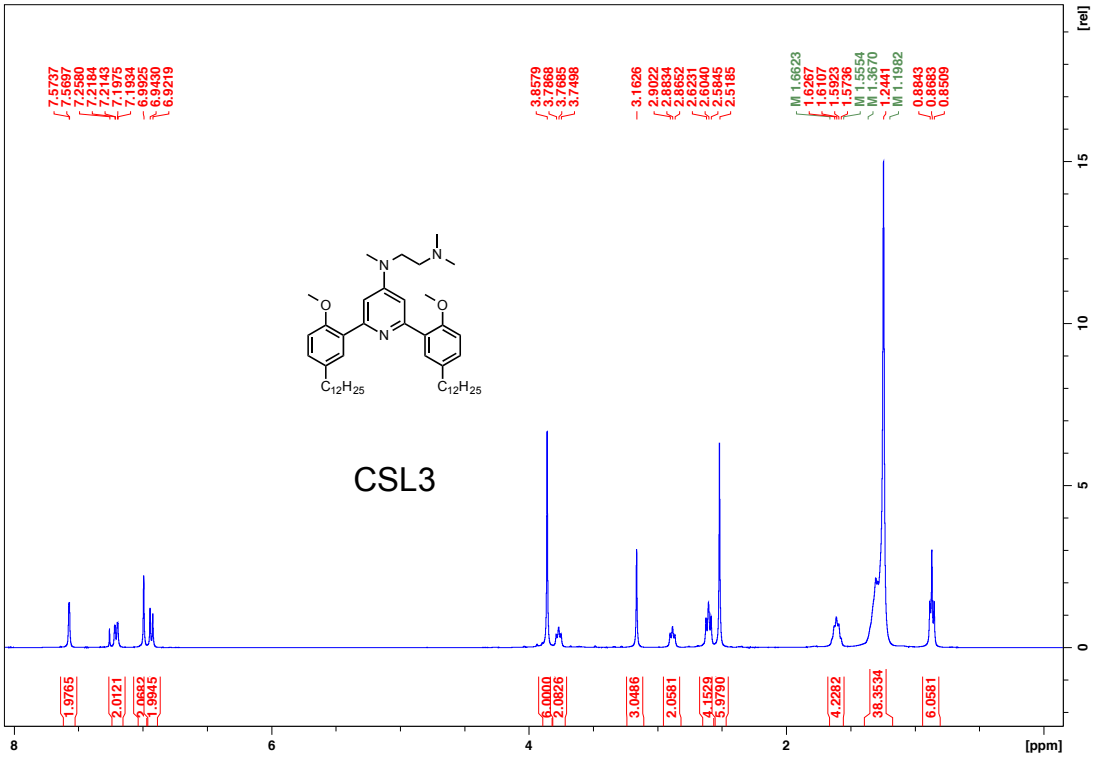


Figure S14. ^{1}HNMR and ^{13}CNMR spectrums of compound CSL3



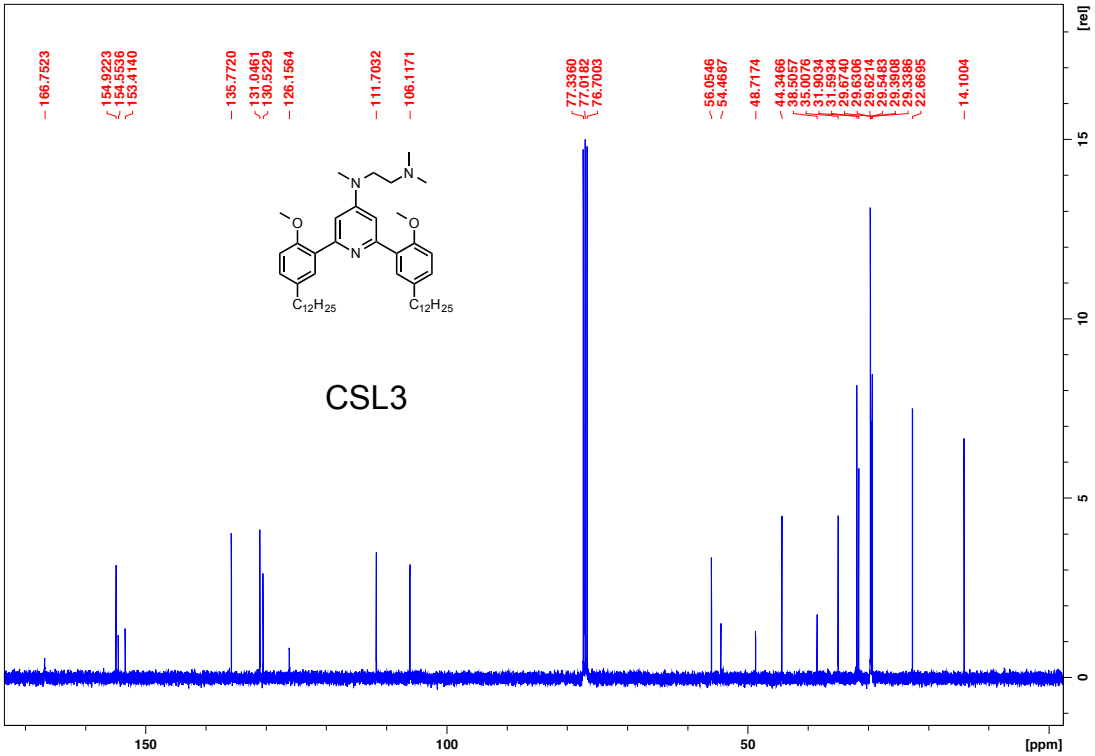
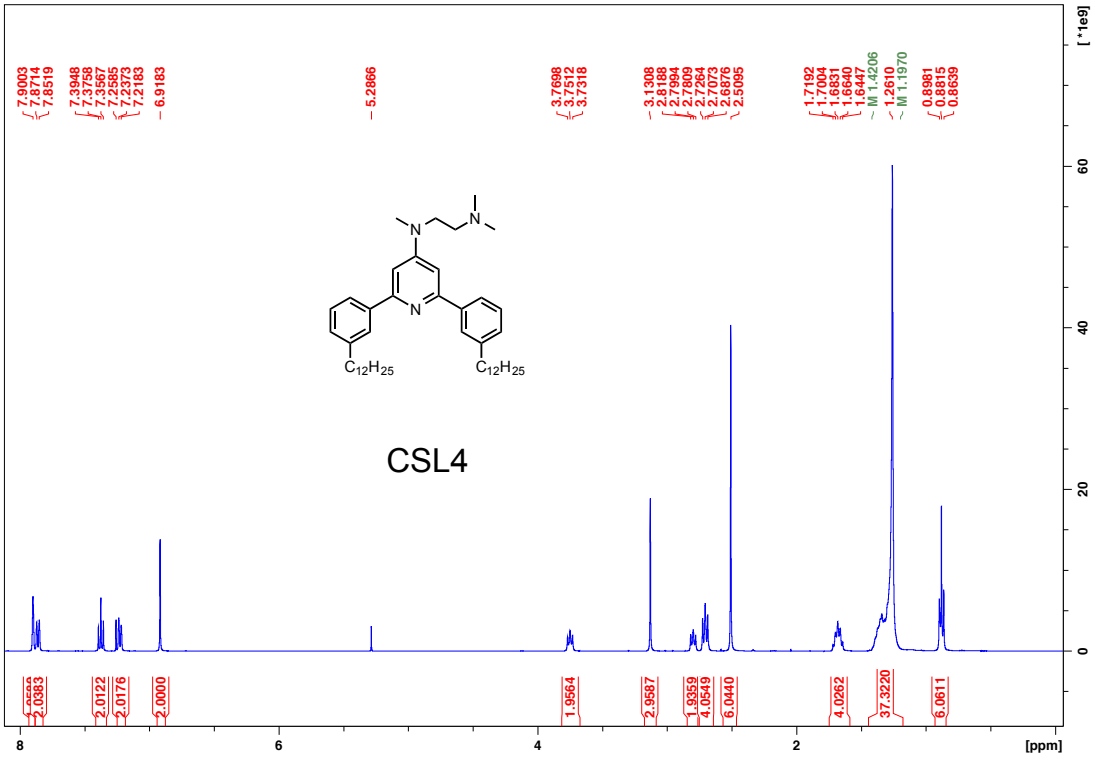
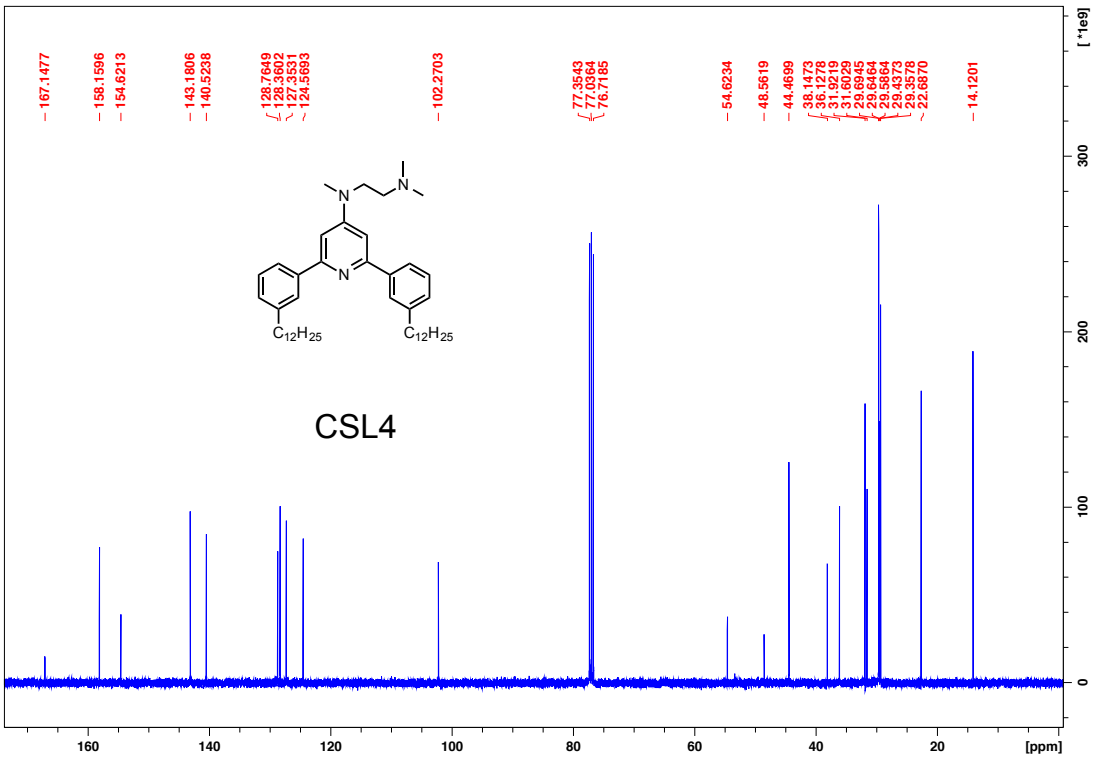


Figure S15. ^{1}HNMR and ^{13}CNMR spectrums of compound CSL4





Materials, general methods and cell culture

All solvents and reagents were obtained from Sigma-Aldrich (Oakville, ON, Canada), Fisher Scientific (Ottawa, ON, Canada), Alfa Aesar (Ward Hill, MA, USA) and Oakwood Chemical (West Columbia, SC, USA). Anhydrous tetrahydrofuran (THF) and dimethylformamide (DMF) were obtained from a Pure Solv PS-400-6 System (Innovative Technology, Amesbury, MA, USA). Cholesterol, DSPC (1,2-distearoyl-sn-glycero-3-phosphocholine) and DSPE-PEG_{2000} (N-(carbonyl-methoxypolyethyleneglycol 2000)-1,2-distearoyl-sn-glycero-3-phosphoethanolamine, sodium salt) were supplied by Avanti® Polar Lipids (Alabaster, AL, USA). DMG-PEG_{2000} (1,2-dimyristoyl-sn-glycerol, methoxypolyethyleneglycol 2000) was purchased from NOF America Corporation (White Plains, NY, USA). Chloroquine, genistein, chlorpromazine and resazurin sodium salt were obtained from Sigma-Aldrich (Oakville, ON, Canada), 5-(N-Ethyl-N-isopropyl)-Amiloride (EIPA) was obtained from Santa Cruz Biotechnology (Dallas, TX, USA), Pitstop 2™ was obtained from Abcam® (Toronto, ON, Canada) and Bafilomycin A1 was obtained from Alfa Aesar (Ward Hill, MA, USA). All solvents and reagents were of analytical grade and were used as received. All liquid nuclear magnetic resonance spectra were recorded on a Varian 400 WB spectrometer, using residual solvent peak for calibration. High-resolution mass spectroscopy analysis was performed by the Regional Center for Mass Spectrometry of the University of Montréal. Chemical reactions were monitored by LC-MS (Infinity 1260 + MS 6120, Agilent Technologies, Mississauga, ON, Canada).

HeLa/GFP cells (GFP Reporter Stable Cell Line, Cell Biolabs Inc., San Diego, CA, USA) and HeLa cells (CCL-2™, ATCC®, Manassas, VA, USA) were cultured in Eagle's Minimum Essential Medium (EMEM, cat #30-2003, ATCC®) supplemented with 10% Fetal Bovine Serum (Gibco, Burlington, ON, Canada). Huh-7 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM, cat# 319-005-CL, Wisent, Montreal, QC, Canada) supplemented with 10% Fetal Bovine Serum (Gibco). Cells were incubated at 37°C under a water-saturated atmosphere supplemented with 5% CO_2.

Statistical analyses were performed using Prism® 6 (GraphPad Software, La Jolla, CA, USA).

Synthesis of the pH-sensitive cationic switchable lipids CSL1 and CSL3

1.1 Synthesis of compounds **1** & **2**

In a 250 mL round-bottom flask previously purged with argon was added sodium hydride (60% dispersion in mineral oil, un-rinsed) (926 mg / 27.6 mmol). Anhydrous THF (130 mL) was added, and 2,6-dibromopyridin-4-amine[4] (1736 mg / 6.89 mmol) was slowly dissolved in the mixture. 2-iodo-*N*,*N*-dimethylethan-1-amine (iodide salt)[5] (2253 mg / 6.89 mmol) was added and the mixture was stirred 0.5 hours at 80°C under argon. The mixture was then slowly quenched with MeOH, and dry loaded into column chromatography on silica gel for purification (hexane/AcOEt, fast gradient from 100:0 to 0:100 ; DCM/MeOH, gradient from 100:0 to 70:30) using a Teledyne Isco CombiFlash® purification system. The final product **1** was obtained as a yellow solid (1386 mg / 62%) and the final product **2** was obtained as a slightly orange solid (395 mg / 15%).

1 : ^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm) 2.22 (s, 6H), 2.52 (t, J = 5.7 Hz, 2H), 3.08 (q, J_1 = 6.2 Hz, J_2 = 5.0 Hz, 2H), 6.56 (s, 2H). ^{13}C NMR (100 MHz, $CDCl_3$) δ (ppm) 39.49, 44.86, 56.68, 109.92, 140.60, 156.02. Calc [M+H] = 321.95490 ; Exp [M+H] = 321.95555 ; Error = - 2.01 ppm.

2 : ^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm) 2.25 (s, 12H), 2.42 (t, J = 7.2 Hz, 4H), 3.37 (t, J = 7.2 Hz, 4H), 6.58 (s, 2H). ^{13}C NMR (100 MHz, $CDCl_3$) δ (ppm) 45.80, 49.22, 55.91, 108.80, 141.06, 155.22. Calc [M+H] = 393.02840 ; Exp [M+H] = 393.02934 ; Error = - 2.41 ppm.

1.2 Synthesis of compound **3**

In a 250 mL round-bottom flask previously purged with argon was added sodium hydride (60% dispersion in mineral oil, un-rinsed) (654 mg / 19.5 mmol). Anhydrous THF (50 mL) was added, and **1** (786 mg / 2.43 mmol) was added to the mixture. Methyl iodide was added (449 mg / 3.16 mmol). The reaction was stirred at 40°C and was carefully monitored by LC-MS until entire consumption of the starting material (~ 2h). The mixture was then slowly quenched with MeOH, filtered (0.20 µm PTFE filter) and dry loaded into column chromatography on silica gel for purification (hexane/AcOEt, fast gradient from 100:0 to 0:100; DCM/MeOH, gradient from 100:0 to 80:20) using a Teledyne Isco CombiFlash® purification system. The final product **3** was obtained as a

yellow oil (640 mg / 78%). ^{1}H NMR (400 MHz, CD_3OD) δ (ppm) 2.30 (s, 6H), 2.49 (t, *J* = 7.4 Hz, 2H), 2.99 (s, 3H), 3.49 (t, *J* = 7.3 Hz, 2H), 6.77 (s, 2H). ^{13}C NMR (100 MHz, CD_3OD) δ (ppm) 37.19, 44.41, 49.10, 54.80, 108.68, 140.19, 156.34. Calc [M+H] = 335.97055 ; Exp [M+H] = 335.97180 ; Error = - 3.71 ppm.

1.3 Synthesis of compound **4**

In a 50 mL round-bottom flask was dissolved **2** (127 mg / 0.32 mmol) and 5-bromo-2-methoxyphenylboronic acid (170 mg / 0.74 mmol) in 4 mL of THF. Na_2CO_3 (137 mg / 1.29 mmol), previously dissolved in 4 mL of distilled water, was added and the flask was purged three times with argon. $Pd(PPh_3)_4$ (3.7 mg / 0.003 mmol) was added and the flask was purged three times again. The mixture was stirred 48 hours at 80°C. The reaction was monitored by LC-MS until entire consumption of the starting materials. The mixture was then diluted with saturated Na_2CO_3 and extracted 3 times with dichloromethane (DCM). The organic layer was dried over $MgSO_4$ and evaporated under vacuum. The crude product was purified by reverse phase preparative HPLC on an Agilent Zorbax PrepHT Eclipse XDB-C18 21.2x150mm (5µm) column. Mobile phase A was composed of water + 0.1% formic acid and mobile phase B was composed of MeOH + 0.1% formic acid. The mobile phase gradient was: 0 min – 15 % B ; 6 min – 80 % B ; 12 min - 80 % B followed by a column re-equilibration time of 4 min. Compound **4** was obtained as a white solid (58 mg / 30%). ^{1}H NMR (400 MHz, CD_3OD) δ (ppm) 2.48 (s, 12H), 2.79 (t, *J* = 6.5 Hz, 4H), 3.67 (t, *J* = 6.5 Hz, 4H), 3.86 (s, 6H), 6.97 (s, 2H), 7.05 (d, *J* = 8.9 Hz, 2H), 7.50 (dd, J_1 = 8.9 Hz, J_2 = 2.6 Hz, 2H), 7.73 (d, *J* = 2.6 Hz, 2H). ^{13}C NMR (100 MHz, CD_3OD) δ (ppm) 44.14, 55.21, 56.08, 107.00, 112.41, 113.35, 130.81, 132.32, 133.06, 152.55, 154.18, 156.23. Calc [M+H] = 605.11213 ; Exp [M+H] = 605.11451 ; Error = - 3.93 ppm.

1.4 Synthesis of compound **5**

In a 100 mL round-bottom flask was dissolved **3** (694 mg / 2.06 mmol) and 5-bromo-2-methoxyphenylboronic acid (1189 mg / 5.15 mmol) in 6 mL of THF. Na_2CO_3 (873 mg / 8.24 mmol), previously dissolved in 6 mL of distilled water, was added and the flask was purged three times with argon. $Pd(PPh_3)_4$ (23.8 mg / 0.021 mmol) was added and the flask was purged three times again. The mixture was stirred 48 hours at 80°C. The reaction was monitored by LC-MS until entire consumption of the starting materials. The mixture was then diluted with

saturated Na_2CO_3 and extracted 3 times with dichloromethane (DCM). The organic layer was dried over $MgSO_4$ and evaporated under vacuum. The final product was purified using a Teledyne Isco CombiFlash® purification system equipped with a 30 grams reversed-phase C18 HP Gold column. Mobile phase A was composed of water + 0.1% formic acid and mobile phase B was composed of MeOH + 0.1% formic acid. The mobile phase gradient was: 0 min – 20 % B ; 15 min – 90 % B followed by a column re-equilibration time of 4-5 CV. Compound **5** was obtained as a white solid (492 mg / 47%). ^{1}H NMR (400 MHz, CD_3OD) δ (ppm) 2.35 (s, 6H), 2.62 (t, *J* = 7.2 Hz, 2H), 3.07 (s, 3H), 3.59 (t, *J* = 7.3 Hz, 2H), 3.84 (s, 6H), 6.94 (s, 2H), 7.02 (d, *J* = 8.8 Hz, 2H), 7.48 (dd, J_1 = 8.8 Hz, J_2 = 2.5 Hz, 2H), 7.70 (d, *J* = 2.5 Hz, 2H). ^{13}C NMR (100 MHz, CD_3OD) δ (ppm) 37.09, 44.29, 48.82, 54.84, 55.17, 106.68, 112.36, 113.35, 130.40, 132.39, 133.07, 153.30, 154.21, 156.25. Calc [M+H] = 548.05428 ; Exp [M+H] = 548.05574 ; Error = - 2.66 ppm.

1.5 Synthesis of compound **CSL1**

Compound **4** (80 mg / 0.132 mmol) was weighted in a 20 mL microwave reaction vial. 1-dodecyne (66 mg / 0.40 mmol) and PPh_3 (10.4 mg / 0.04 mmol) were added. Dried tetra-n-butylammonium fluoride (TBAF) (241 mg / 0.92 mmol) was added (previously dried under high vacuum from 1.0 M TBAF solution in THF), followed by 2 mL of anhydrous DMF and 2 mL of diisopropylamine (DIPA). The reaction vial was purged 3 times with argon. $PdCl_2(PPh_3)_2$ was added (8.3 mg / 0.01 mmol), the vial was purged 3 times with argon and sealed. The mixture was stirred 16 hours at 80°C. The mixture was then diluted with saturated Na_2CO_3 and extracted 3 times with DCM. The organic layer was dried over $MgSO_4$ and evaporated under vacuum. The crude product was purified by column chromatography on silica gel (DCM/MeOH, gradient from 100:0 to 70:30) using a Teledyne Isco CombiFlash® purification system. The product was then dissolved in EtOH in a 100 mL round-bottom flask. Palladium (10 wt %Pd basis) on activated charcoal (40 mg) was added and the flask was purged 3 times with hydrogen. The mixture was then stirred for 4 hours at room temperature under 1 atm hydrogen pressure. The solution was then filtered on a PTFE filter (0.20 µm) to remove the catalyst, and the organic phase was evaporated under vacuum. The final product was purified by reverse phase preparative HPLC on an Agilent Zorbax PrepHT Eclipse XDB-C8 21.2x100mm (5µm) column. Mobile phase A was composed of water + 0.1% formic acid and mobile phase B was composed of MeOH + 0.1% formic acid. The mobile phase gradient was: 0 min – 30 % B ; 10 min – 95 % B ; 12 min - 95 % B followed by a column re-equilibration time of 4 min.

Compound **CSL1** was obtained as a white solid (40 mg / 38%). ^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm) 0.87 (t, *J* = 6.6 Hz, 6H), 1.19-1.38 (m, 36H), 1.62 (quint, *J* = 7.4 Hz, 4H), 2.34 (s, 12H), 2.58-2.63 (m, 8H), 3.53 (t, *J* = 7.4 Hz, 4H), 3.84 (s, 6H), 6.89 (d, *J* = 8.4 Hz, 2H), 7.00 (s, 2H), 7.12 (dd, J_1 = 8.4 Hz, J_2 = 2.3 Hz, 2H), 7.67 (d, *J* = 2.3 Hz, 2H). ^{13}C NMR (100 MHz, $CDCl_3$) δ (ppm) 14.10, 22.68, 29.35-29.68, 29.70, 31.62, 35.12, 45.57, 48.61, 55.91, 56.06, 106.39, 111.42, 128.91, 130.19, 131.43, 135.30, 152.10, 154.96, 155.89. Calc [M+H] = 785.6667 ; Exp [M+H] = 785.66949 ; Error = - 3.54 ppm.

1.6 Synthesis of compound **CSL3**

Compound **5** (228 mg / 0.42 mmol) was weighted in a 20 mL microwave reaction vial. 1-dodecyne (207 mg / 1.25 mmol) and PPh_3 (33.7 mg / 0.125 mmol) were added. Dried tetra-n-butylammonium fluoride (TBAF) (760 mg / 2.91 mmol) was added (previously dried under high vacuum from 1.0 M TBAF solution in THF), followed by 2 mL of anhydrous DMF and 2 mL of diisopropylamine (DIPA). The reaction vial was purged 3 times with argon. $PdCl_2(PPh_3)_2$ was added (26 mg / 0.037 mmol), the vial was purged 3 times with argon and sealed. The mixture was stirred 16 hours at 80°C. The mixture was then diluted with saturated Na_2CO_3 and extracted 3 times with DCM. The organic layer was dried over $MgSO_4$ and evaporated under vacuum. The crude product was purified by column chromatography on silica gel (DCM/MeOH, gradient from 100:0 to 90:10) using a Teledyne Isco CombiFlash® purification system. The product was then dissolved in EtOH in a 100 mL round-bottom flask. Palladium (10 wt %Pd basis) on activated charcoal (50 mg) was added and the flask was purged 3 times with hydrogen. The mixture was then stirred 4 hours at room temperature under a 1 atm hydrogen pressure. The solution was then filtered on a PTFE filter (0.20 µm) to remove the catalyst, and the organic phase was evaporated under vacuum. The final product was purified by reverse phase preparative HPLC on an Agilent Zorbax PrepHT Eclipse XDB-C8 21.2x100mm (5µm) column. Mobile phase A was composed of water + 0.1% formic acid and mobile phase B was composed of MeOH + 0.1% formic acid. The mobile phase gradient was: 0 min – 30 % B ; 10 min – 95 % B ; 12 min - 95 % B followed by a column re-equilibration time of 4 min. Compound **CSL3** was obtained as a slightly yellow wax (134 mg / 44%). ^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm) 0.87 (t, *J* = 6.4 Hz, 6H), 1.20-1.37 (m, 36H), 1.59 (quint, *J* = 7.4 Hz, 4H), 2.52 (s, 6H), 2.60 (t, *J* = 7.5 Hz, 4H), 2.88 (t, *J* = 7.5 Hz, 2H), 3.16 (s, 3H), 3.77 (t, *J* = 7.5 Hz, 2H), 3.86 (s, 6H), 6.93 (d, *J* = 8.4 Hz, 2H), 6.99 (s, 2H), 7.20 (dd, J_1 = 8.4 Hz, J_2 = 2.2 Hz, 2H), 7.57 (d, *J* = 2.2 Hz, 2H). ^{13}C NMR (100 MHz, $CDCl_3$) δ (ppm) 14.10, 22.67, 29.34-29.63,

29.67, 31.59, 35.00, 38.51, 44.35, 48.72, 54.47, 56.05, 106.12, 111.70, 126.16, 130.52, 131.05, 135.77, 153.41, 154.55, 154.92. Calc [M+H] = 728.60886 ; Exp [M+H] = 728.61108 ; Error = - 3.06 ppm.

Synthesis of the pH-sensitive cationic switchable lipid CSL2

2.1 Synthesis of compound **6**

Ethylenediamine (640 mg / 10.64 mmol) was added dropwise to a suspension of sodium hydride (60% dispersion in mineral oil, un-rinsed) (255 mg / 10.64 mmol) in anhydrous DMF (20 mL). 2,6-dibromo-4-nitro-pyridine[6] (2 g / 7.10 mmol) was introduced by small amounts. The mixture was stirred at room temperature for 3 hours, then carefully quenched with MeOH, washed with saturated Na_2CO_3 and extracted with AcOEt. The organic layer was dried over $MgSO_4$ and concentrated to afford a brown oil. The crude was purified by chromatography on silica gel (DCM/MeOH, gradient from 100:0 to 80:20) using a Teledyne Isco CombiFlash® purification system to afford **6** (628 mg / 30%) as an orange oil. ^{1}H NMR (400 MHz, CD_3OD) δ (ppm) 2.80 (t, *J* = 7.0 Hz, 2H), 3.18 (t, *J* = 7.0 Hz, 2H), 6.70 (s, 2H). ^{13}C NMR (100 MHz, CD_3OD) δ (ppm) 40.81, 45.12, 110.61, 141.29, 158.69. Calc [M+H] = 293.92360 ; Exp [M+H] = 293.92232 ; Error = - 4.37 ppm.

2.2 Synthesis of compound **7**

To a solution of **6** (1.5 g / 5.08 mmol) in DCM (25 mL) was slowly added Boc_2O (1.33 g / 6.10 mmol). The medium was stirred at room temperature during 2h and then washed with saturated Na_2CO_3. The organic layer was decanted and the aqueous layer was extracted twice with DCM. The combined organic layers were dried over $MgSO_4$ and concentrated under reduced pressure to afford a brown oil. The crude was purified by chromatography on silica gel (hexane/AcOEt, gradient from 100:0 to 0:100) using a Teledyne Isco CombiFlash® purification system to afford **7** (1.6 g / 79%) as an orange oil. ^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm) 1.44 (s, 9H), 3.20 (m, 2H), 3.38 (m, 2H), 4.92 (t, J = 2.4 Hz, 1H), 5.54 (br, 1H), 6.54 (s, 2H). ^{13}C NMR (100 MHz, $CDCl_3$) δ (ppm) 28.26, 39.58, 44.65, 80.43, 109.69, 140.66, 156.24, 157.40. Calc [M+H] = 393.97603 ; Exp [M+H] = 393.97785 ; Error = 4.6 ppm.

2.3 Synthesis of compound **8**

To a solution of **7** (1.2 g, 3.04 mmol) in anhydrous DMF (10 mL) was added sodium hydride (60% dispersion in mineral oil, un-rinsed) (153 mg / 4.56 mmol) and methyl iodide (863 mg, 6.08 mmol). The mixture was stirred at 40°C and was monitored by LC-MS until entire consumption of the starting material (~ 3h). The mixture was then slowly quenched with MeOH, washed with water and extracted with DCM. The crude was dry loaded into column chromatography on silica gel for purification (hexane/AcOEt, gradient from 100:0 to 0:100) using a Teledyne Isco CombiFlash® purification system to afford **8** (907 mg / 73%) as an orange solid. ^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm) 1.42 (s, 9H), 2.97 (s, 3H), 3.29 (m, 2H), 3.47 (m, 2H), 4.70 (br, 1H), 6.65 (s, 2H). ^{13}C NMR (100 MHz, $CDCl_3$) δ (ppm) 25.76, 35.30, 48.64, 77.40, 106.30, 138.46, 153.49, 153.72. Calc [M+H] = 407.99168 ; Exp [M+H] = 407.99221 ; Error = 1.3 ppm.

2.4 Synthesis of compound **9**

In a 50 mL round-bottom flask was dissolved **8** (240 mg / 0.59 mmol) and 5-bromo-2-methoxyphenylboronic acid (340 mg / 1.47 mmol) in 5 mL of THF. Na_2CO_3 (249 mg / 2.35 mmol), previously dissolved in 5 mL of distilled water, was added and the flask was purged three times with argon. $Pd(PPh_3)_4$ (8.2 mg / 0.012 mmol) was added and the flask was purged three times again. The mixture was stirred 48 hours at 80°C. The reaction was monitored by LC-MS until entire consumption of the starting materials. The mixture was then diluted with saturated Na_2CO_3 and extracted 3 times with dichloromethane (DCM). The organic layer was dried over $MgSO_4$ and evaporated under vacuum. The crude was dry loaded into column chromatography on silica gel for purification (hexane/AcOEt, gradient from 100:0 to 0:100) using a Teledyne Isco CombiFlash® purification system to afford **9** (179 mg / 49%) as an orange solid. ^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm) 1.37 (s, 9H), 3.09 (s, 3H), 3.38 (m, 2H), 3.60 (m, 2H), 3.85 (s, 6H), 4.79 (br, 1H), 6.86 (d, J = 8.7 Hz, 2H), 6.95 (s, 2H), 7.44 (dd, J_1 = 8.7 Hz, J_2 = 2.5 Hz, 2H), 7.84 (d, J = 2.5 Hz, 2H). ^{13}C NMR (100 MHz, $CDCl_3$) δ (ppm) 28.37, 38.31, 51.25, 56.22, 79.42, 106.69, 113.22, 113.29, 131.01, 132.44, 133.75, 153.62, 156.11, 164.74. Calc [M+H] = 620.07541 ; Exp [M+H] = 620.07685 ; Error = 2.3 ppm.

2.5 Synthesis of compound **10**

Compound **9** (270 mg / 0.34 mmol) was weighted in a 20 mL microwave reaction vial. 1-dodecyne (113 mg / 1.30 mmol) and PPh_3 (20 mg / 0.078 mmol) were added. Dried tetra-n-butylammonium fluoride (TBAF) (355 mg / 2.60 mmol) was added (previously dried under high vacuum from 1.0 M TBAF solution in THF), followed by 1 mL of anhydrous DMF and 1 mL of diisopropylamine (DIPA). The reaction vial was purged 3 times with argon. $PdCl_2(PPh_3)_2$ was added (21 mg / 0.030 mmol), the vial was purged 3 times with argon and sealed. The mixture was stirred 16 hours at 80°C. The mixture was then diluted with saturated Na_2CO_3 and extracted 3 times with DCM. The organic layer was dried over $MgSO_4$ and evaporated under vacuum. The crude product was purified by column chromatography on silica gel (hexane/AcOEt, gradient from 100:0 to 0:100) using a Teledyne Isco CombiFlash® purification system to afford **10** (141 mg / 41%) as a yellow oil. ^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm) 0.87 (t, *J* = 6.8 Hz, 6H), 1.16-1.34 (m, 28H), 1.35-1.50 (m, 13H), 1.58 (quint, *J* = 7.6 Hz, 4H), 2.37 (t, *J* = 7.2 Hz, 4H), 3.05 (s, 3H), 3.36 (m, 2H), 3.54 (m, 2H), 3.84 (s, 6H), 4.69 (br, 1H), 6.88 (d, *J* = 8.6 Hz, 2H), 6.92 (s, 2H), 7.35 (dd, J_1 = 8.6 Hz, J_2 = 2.4 Hz, 2H), 7.80 (d, *J* = 2.4 Hz, 2H). ^{13}C NMR (100 MHz, $CDCl_3$) δ (ppm) 14.10, 19.45, 22.67, 28.27, 28.91-29.60, 31.89, 38.08, 38.21, 51.00, 55.80, 80.36, 88.83, 106.55, 111.27, 116.60, 130.66, 132.44, 134.66, 155.42, 156.32. Calc [M+H] = 792.56738 ; Exp [M+H] = 792.5681 ; Error = 0.91 ppm.

2.6 Synthesis of compound **CSL2**

Compound **10** (141 mg / 0.179 mmol) was dissolved in EtOH in a 100 mL round-bottom flask. Palladium (10 wt %Pd basis) on activated charcoal (50 mg) was added and the flask was purged 3 times with hydrogen. The mixture was then stirred 4 hours at room temperature under a 1 atm hydrogen pressure. The solution was then filtered on a PTFE filter (0.20 µm) to remove the catalyst, and the organic phase was evaporated under vacuum. The oily crude was dissolved in 4M HCl in dioxane (2 mL) and was stirred 2 hours at room temperature. The mixture was then slowly diluted with saturated Na_2CO_3 and extracted 3 times with DCM. The organic layer was dried over $MgSO_4$ and evaporated under vacuum. The final product was purified by reverse phase preparative HPLC on an Agilent Zorbax PrepHT Eclipse XDB-C8 21.2x100mm (5µm) column. Mobile phase A was composed of water + 0.1% formic acid and mobile phase B was composed of MeOH + 0.1% formic acid. The mobile phase gradient was: 0 min – 30 % B ; 10 min – 95 % B ; 12 min - 95 % B followed by a column re-

equilibration time of 4 min. Compound **CSL2** was obtained as a white waxy solid (80 mg / 64%). ^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm) 0.87 (t, *J* =7.0 Hz, 6H), 1.18-1.37 (m, 36H), 1.59 (quint, *J* = 7.5 Hz, 4H), 2.59 (t, *J* = 7.8 Hz, 4H), 3.10 (s, 3H), 3.71 (m, 2H), 3.82 (s, 6H), 6.89 (d, *J* = 8.4 Hz, 2H), 7.04 (s, 2H), 7.14 (dd, J_1 = 8.4 Hz, J_2 = 2.3 Hz, 2H), 7.63 (d, *J* = 2.3 Hz, 2H). ^{13}C NMR (100 MHz, $CDCl_3$) δ (ppm) 14.11, 22.68, 29.35-29.69, 31.62, 31.91, 35.06, 38.51, 56.00, 106.31, 111.64, 127.87, 129.80, 131.14, 135.60, 154.23, 154.30, 154.96. Calc [M+H] = 700.57756 ; Exp [M+H] = 700.57702 ; Error = - 0.8 ppm.

Synthesis of the negative control cationic lipid CSL4

3.1 Synthesis of compound **11**

In a 50 mL round-bottom flask was dissolved **3** (171 mg / 0.51 mmol) and 3-bromophenylboronic acid (234 mg / 1.17 mmol) in 4 mL of THF. Na_2CO_3 (215 mg / 2.03 mmol), previously dissolved in 4 mL of distilled water, was added and the flask was purged three times with argon. $Pd(PPh_3)_4$ (6.0 mg / 0.005 mmol) was added and the flask was purged three times again. The mixture was stirred 24 hours at 80°C. The reaction was monitored by LC-MS until entire consumption of the starting materials. The mixture was then diluted with saturated Na_2CO_3 and extracted 3 times with dichloromethane (DCM). The organic layer was dried over $MgSO_4$ and evaporated under vacuum. The final product was purified by reverse phase preparative HPLC on an Agilent Zorbax PrepHT Eclipse XDB-C18 21.2x150mm (5µm) column. Mobile phase A was composed of water + 0.1% formic acid and mobile phase B was composed of MeOH + 0.1% formic acid. The mobile phase gradient was: 0 min – 15 % B ; 6 min – 80 % B ; 12 min - 80 % B followed by a column re-equilibration time of 4 min. Compound **4** was obtained as a slightly yellow waxy solid (139 mg / 56%). ^{1}H NMR (400 MHz, CD_3OD) δ (ppm) 2.80 (s, 6H), 3.03 (s, 3H), 3.15 (t, *J* = 2.3 Hz, 2H), 3.78 (t, *J* = 2.3 Hz, 2H), 6.92 (s, 2H), 7.33 (t, *J* = 7.7 Hz, 2H), 7.52 (d, *J* = 7.6 Hz, 2H), 7.95 (d, *J* = 7.7 Hz, 2H), 8.19 (s, 2H). ^{13}C NMR (100 MHz, CD_3OD) δ (ppm) 38.13, 43.68, 47.46, 54.19, 104.01, 123.58, 126.98, 131.04, 131.30, 132.77, 143.14, 156.30, 156.93. Calc [M+H] = 488.03315 ; Exp [M+H] = 488.03532 ; Error = 4.4 ppm.

3.2 Synthesis of compound **CSL4**

Compound **11** (139 mg / 0.28 mmol) was weighted in a 10 mL microwave reaction vial. 1-dodecyne (139 mg / 0.83 mmol) and PPh_3 (22 mg / 0.083 mmol) were added. Dried tetra-n-butylammonium fluoride (TBAF) (509 mg / 1.95 mmol) was added (previously dried under high vacuum from 1.0 M TBAF solution in THF), followed by 1 mL of anhydrous DMF and 1 mL of diisopropylamine (DIPA). The reaction vial was purged 3 times with argon. $PdCl_2(PPh_3)_2$ was added (17 mg / 0.025 mmol), the vial was purged 3 times with argon and sealed. The mixture was stirred 16 hours at 80°C. The mixture was then diluted with saturated Na_2CO_3 and extracted 3 times with DCM. The organic layer was dried over $MgSO_4$ and evaporated under vacuum. The crude product was purified by column chromatography on silica gel (DCM/MeOH, gradient from 100:0 to 90:10) using a Teledyne Isco CombiFlash® purification system. The product was then dissolved in EtOH in a 100 mL round-bottom flask. Palladium (10 wt %Pd basis) on activated charcoal (50 mg) was added and the flask was purged 3 times with hydrogen. The mixture was then stirred 4 hours at room temperature under a 1 atm hydrogen pressure. The solution was then filtered on a PTFE filter (0.20 µm) to remove the catalyst, and the organic phase was evaporated under vacuum. The final product was purified by reverse phase preparative HPLC on an Agilent Zorbax PrepHT Eclipse XDB-C8 21.2x100mm (5µm) column. Mobile phase A was composed of water + 0.1% formic acid and mobile phase B was composed of MeOH + 0.1% formic acid. The mobile phase gradient was: 0 min – 30 % B ; 10 min – 95 % B ; 12 min - 95 % B followed by a column re-equilibration time of 4 min. Compound **CSL4** was obtained as a colorless oil (45 mg / 24%). ^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm) 0.88 (t, *J* = 6.6 Hz, 6H), 1.20-1.42 (m, 36H), 1.68 (quint, *J* = 7.5 Hz, 4H), 2.51 (s, 6H), 2.71 (t, *J* = 7.5 Hz, 4H), 2.80 (t, *J* = 7.5 Hz, 2H), 3.13 (s, 3H), 3.75 (t, *J* = 7.6 Hz, 2H), 6.92 (s, 2H), 7.22 (d, *J* = 7.6 Hz, 2H), 7.38 (t, *J* = 7.6 Hz, 2H), 7.86 (d, *J* = 7.6 Hz, 2H), 7.90 (s, 2H). ^{13}C NMR (100 MHz, $CDCl_3$) δ (ppm) 14.12, 22.69, 29.36-29.69, 31.60, 31.92, 36.13, 38.15, 44.47, 48.56, 54.62, 102.27, 124.57, 127.35, 128.36, 128.76, 140.52, 143.18, 154.62, 158.16, 167.15. Calc [M+H] = 668.58773 ; Exp [M+H] = 668.58994 ; Error = 3.31 ppm.

Lipid nanoparticle preparation by manual extrusion

Lipid nanoparticles for *in vitro* studies were prepared by manual extrusion, using cationic switchable lipids (CSL), DSPC, cholesterol and DSPE-PEG_{2000} at a molar ratio of 50:10:37.5:2.5 respectively. For *in vivo* experiments, lipid nanoparticles were prepared using microfluidic mixing (further detailed below). Stock solutions (20-40 mg/mL) of cationic switchable lipids and commercial co-lipids were prepared in ethanol and stored at -80°C before use. The lipid stock solutions were combined in a 5 mL round-bottom flask at the desired molar ratio, and the ethanol was removed under reduced pressure. The lipidic film thus obtained was dried overnight under high vacuum to remove any residual solvent. The lipidic film was hydrated on a vortex mixer with 1 mL of sterile 5% dextrose in water. The lipid suspension was then subjected to stepwise extrusions through polycarbonate membranes (200 and 100 nm – 9 passages per membrane) using a LiposoFast manual extruder (Avestin Inc., Ottawa, ON, Canada) heated at 60°C. The cationic switchable lipid amount present in each preparation was quantified *via* HPLC-UV/MS (1260 Infinity, Agilent Technologies) against a calibration curve of cationic switchable lipid (25-250 µg/mL; from ethanol stock solution).

siRNA complexation and encapsulation efficiency

Stock solutions of lipid nanoparticle (made by manual extrusion) and siRNA were diluted in sterile 5% dextrose at appropriate concentrations, depending upon the lipid nitrogen/siRNA phosphate (N/P) ratio desired (unless indicated otherwise, a N/P ratio value of 4 was used for all the experiments). siRNA solution was added to the lipid nanoparticle solution, followed by brief vortexing. Complexation was then realized during 15 minutes at 50°C under vigorous vortexing (1200rpm) in a Labnet Vortemp™ 56.

For quantification of the encapsulation efficiency, lipid nanoparticles and siRNA were prepared for a final siRNA concentration of 100 nM. A SYBR® Gold (Thermo Scientific) assay was used to quantify the siRNA encapsulation efficiency of the formulations. After complexation (15 minutes, 50°C, vigorous vortexing), the solutions were centrifuged at 20 000*g* for 30 minutes. Unencapsulated free siRNA in the supernatant was quantified against a calibration curve of siRNA (2-100 nM), using the SYBR® Gold fluorescent dye ($\lambda_{ex/em}$ = 495/537) and a Safire microplate reader (Tecan, Seestrasse, Switzerland). siRNA encapsulation efficiency (%) was calculated as follows:

$$EE\ (\%) = \frac{100\ (nM)\ -\ siRNA\ concentration\ in\ the\ supernatant\ (nM)}{100\ (nM)} x100$$

Physicochemical characterization of lipid nanoparticles

Hydrodynamic diameter and ζ-potential of lipid nanoparticles/siRNA complexes were measured at 20°C by Dynamic Light Scattering using a Malvern Zetasizer Nano ZS (Malvern, Worcestershire, UK), using the automatic algorithm mode. For size measurements, lipid nanoparticles and siRNA were complexed together (260 nM final siRNA; 200 µL final volume) in 5% dextrose as described above. Complexes were then diluted with 800 µL Opti-MEM®, equilibrated for 20 minutes at 20°C and measured. Size measurements are reported using the Z-Average value. ζ-potential measurements were realized at 20°C using the Smoluchowski model. Lipid nanoparticles and siRNA are complexed together (260 nM final siRNA; 200 µL final volume) in 5% dextrose as described previously. Complexes are then diluted with 800 µL dextrose 5%, equilibrated 15 minutes at 20°C and measured. Experiments were run in triplicate or more.

In vitro siRNA transfection assays

The RNAi induced silencing capabilities of each cationic switchable lipid was investigated in a HeLa/GFP model. Lipid nanoparticles (made by manual extrusion) and anti-GFP siRNA were complexed (N/P ratio of 4) as described above (for a final siRNA concentration/well of 1-60 nM) and were diluted in Opti-MEM® (250 µL). This reverse transfection media (300 µL) was put in the well (12-well plates), and 1 mL of cell suspension in complete culture media (EMEM/FBS 90:10) was added for a final concentration of 40 000 cells/well. Cells were then incubated for 72 hours. After incubation, cells were rinsed with PBS, trypsinized and suspended in FACS buffer (95% PBS, 5% FBS, 1.0 mM EDTA) for immediate analysis on a FACSCalibur™ flow cytometer (BD Biosciences, San Jose, CA, USA). GFP expression for each replicate (mean fluorescent intensity; FlowJo software vX.0.7, Ashland, OR, USA) was calculated relative to the control samples that did not receive any siRNA treatment. Negative control included cells that were treated with naked anti-GFP siRNA (60 nM). Positive control included cells that were treated with Lipofectamine® RNAiMAX (Thermo Scientific) according to the manufacturer's reverse transfection protocol (1 µL reagent/well; 60 nM siRNA/well). All experiments were realized in triplicate.

The RNAi silencing capabilities of the formulations made by microfluidic mixing (aimed for *in vivo* siRNA delivery) was assayed in HeLa/GFP (anti-GFP siRNA) and Huh-7 cells (anti-PCSK9 siRNA), using the same protocol. Transfection efficiency was assayed by either flow cytometry or western blotting[7] after a 48 hour (HeLa/GFP) or 72 hour (Huh-7) incubation period.

Forward transfection was also realized to make sure that transfection efficiency of CSL3 and CSL4 lipid nanoparticles remains similar, regardless of the chosen transfection method. HeLa/GFP cells were seeded in 12-well plates (40 000 cells/well) and allowed to attach overnight. The next day, cells were incubated in Opti-MEM® for 4 hours with lipid nanoparticles (made by manual extrusion) or Lipofectamine® RNAiMAX (1 µL reagent/well; 25 nM siRNA/well). After incubation, cells were rinsed with Opti-MEM® and 1 mL of complete culture media (EMEM/FBS 90:10) was added. Cells were then incubated for 44 hours. After incubation, cells were rinsed with PBS, trypsinized and suspended in FACS buffer for immediate analysis *via* flow cytometry.

In vitro cytotoxicity assay

Cytotoxicity of the lipid nanoparticles (made by manual extrusion) on HeLa/GFP cells was assessed using a resazurin-based cell viability assay (AlamarBlue®). The transfection procedure was strictly identical to that presented above (N/P ratio of 4, 1-60 nM final siRNA concentration/well), but the assay was carefully scaled down from 12-well plates to 96-well plates, according to the growth area of the wells (3000 cells/well, 200 µL final volume/well). After 72 hours of incubation, cells were washed with PBS, and 200 µL of fresh culture medium was added into the wells (EMEM/FBS 90:10). 20 µL of a freshly prepared resazurin solution (440 µM in PBS) were then added, and cells were incubated for 2 hours. The absorbance of each well was measured at 570 and 600 nm using a Safire microplate reader (Tecan). Cellular viability was normalized relative to the negative control (cells treated with dextrose 5%). Experiments were run in sixplicata.

Intracytoplasmic delivery of siRNA

Live-cell fluorescence microscopy was used to image the intracytoplasmic delivery of siRNA-Alexa647 (Qiagen) following transfection with the CSL3-based formulation (CSL3/DSPC/cholesterol/DSPE-PEG_{2000} 50:10:37.5:2.5 mol% - made by manual extrusion). HeLa cells were routinely incubated 24 hours before imaging in 35 mm poly-d-lysine coated glass dishes (MatTek Corporation, Ashland, MA, USA) at a density of 40 000 cells/dish. siRNA were complexed with lipid nanoparticles (N/P ratio of 4) in 5% dextrose as described previously (70 µL final volume). Complexes were then diluted with 346 µL Opti-MEM® and 1384 µL of complete culture media (Opti-MEM®/FBS 90:10), for a final siRNA concentration of 50 nM. Cells were then incubated 10 minutes, 1 hour or 2 hours with the lipid nanoparticles. After incubation, cells were rinsed four times with Opti-MEM®/FBS 90:10 and were immediately imaged in a phenol red-free culture media (Opti-MEM®/FBS 90:10). Imaging of HeLa cells was performed using an Olympus IX81 fluorescent microscope equipped with a Plan Apo N 60X 1.42 NA silicone objective (Olympus Canada Inc., Toronto, ON, Canada) and a 12 bits Retiga-2000R CCD Camera (QImaging, Surrey, BC, Canada), using MetaMorph Advanced software 7.8.9 (Molecular Devices, San Jose, CA, USA). siRNA-Alexa647 was imaged using the Cy5 channel ($\lambda_{ex/em}$ 649/666). All fluorescence images were carefully exported with constant scaling and range of greylevel. Experiment was realized in triplicate.

Endosomal entrapment of the CSL4-based formulation

Live-cell fluorescence microscopy was used to compare the intracellular fate following endocytosis of the CSL3-based (pH-sensitive) and the CSL4-based (negative control) lipid nanoparticles formulations (CSL/DSPC/cholesterol/DSPE-PEG_{2000} 50:10:37.5:2.5 mol% - made by manual extrusion). HeLa cells were routinely incubated 24 hours before imaging in 35 mm poly-d-lysine coated glass dishes (MatTek Corporation) at a density of 40 000 cells/dish. siRNA-Alexa647 (Qiagen) were complexed with lipid nanoparticles (N/P ratio of 4) in 5% dextrose as described previously (70 µL final volume). Complexes were then diluted with 346 µL Opti-MEM® and 1384 µL of complete culture media (Opti-MEM®/FBS 90:10), for a final siRNA concentration of 50 nM. Cells were then incubated 16 hours with the lipid nanoparticles. After incubation, cells were rinsed twice with Opti-MEM®/FBS 90:10 and were stained with Hoechst 33342 (5 min, 2 µg/mL, Sigma). Cells were rinsed twice with Opti-MEM®/FBS 90:10 and were immediately imaged in a phenol red-free culture media (Opti-MEM®/FBS 90:10). Alternatively, cells were exposed to 500 µM chloroquine during the last hour of the 16-hour incubation period (CSL4-based formulation). Imaging of HeLa cells was performed using an Olympus IX81 fluorescent microscope equipped with a UPlanSApo 100X 1.40 NA silicone objective (Olympus Canada Inc.) and a 12 bits Retiga-2000R CCD Camera (QImaging), using MetaMorph Advanced software 7.8.9 (Molecular Devices). Hoechst 33342 was imaged using the DAPI channel ($\lambda_{ex/em}$ 350/470) and siRNA-Alexa647 was imaged using the Cy5 channel ($\lambda_{ex/em}$ 649/666). All fluorescence images were carefully exported with constant scaling and range of greylevel. Experiment was realized in triplicate.

Comparison of the cellular uptake of the CSL3 and CSL4-based formulations over the 16-hour incubation period was assayed by flow cytometry. HeLa cells were seeded in 12-well plates (100 000 cells/well) and allowed to attach overnight. The next day, lipid nanoparticles (25 µL in dextrose 5%) and siRNA-Alexa488 (25 µL in dextrose 5%) were complexed in dextrose 5% (N/P ratio of 4; siRNA concentration 25 nM/well) and were diluted in Opti-MEM® (250 µL) and 1 mL of complete culture media (EMEM/FBS 90:10). The solution was then transferred onto the cells (1300 µL final volume/well) and the plate was incubated for 16 hours. After incubation, cells were rinsed three times with EMEM/FBS 90:10, once with PBS, trypsinized and suspended in FACS buffer (95% PBS, 5% FBS, 1.0 mM EDTA) for immediate analysis *via* flow cytometry. Experiment was realized in triplicate.

Lipid-mixing assay

The fusogenic potential of the CSL3/siRNA and CSL4/siRNA lipid nanoparticles was evaluated with an octadecyl Rhodamine B (R18) lipid-mixing assay.[1,8] R18 was incorporated into the lipid nanoparticles at a self-quenched concentration of 6 mol%. Unlabeled 130 nm POPC vesicles were prepared in 5% dextrose using the manual extrusion method previously described (9 passages through a 200 nm polycarbonate membrane). R18 labeled lipid nanoparticles (CSL/DSPC/cholesterol/DSPE-PEG_{2000}/R18 50:10:31.5:2.5:6 mol% - made by manual extrusion) were complexed with anti-GFP siRNA as described above (N/P ratio of 4, in 5% dextrose). These labeled lipid nanoparticles were mixed with unlabeled POPC vesicles in a buffer at pH 7.4 (5 mM HEPES with ionic strength adjusted to 150 mM with NaCl) or at pH 5 (50 mM acetic buffer with ionic strength adjusted to 150 mM with NaCl), as described below.

In 3000 µL of buffer containing unlabeled POPC vesicles (100 µM total lipid content in the cuvette) were added the labeled lipid nanoparticles/siRNA (50 µL volume; 10 µM total lipid content in the cuvette). The increasing fluorescence of R18 was monitored ($\lambda_{ex/em}$ 556/590, slits 5 nm) using a Hitachi F-2710 Spectrophotometer equipped with a water circulated cell holder with stirring. All experiments were performed at 20°C under medium stirring. After 15 minutes, 4 µL of pure octaethylene glycol monododecyl ether (Sigma) detergent (2.5 mM final concentration in the cuvette) was added to obtain the 100% dequenched fluorescence intensity. Raw data were recorded continuously during the experiment. The percentage of membrane fusion activity at a given time *t* was defined as:

$$\% \, fusion_t = \left(\frac{I_t - I_o}{I_{100\%} - I_o} \right) x \, 100$$

where I_o is the initial fluorescence intensity observed immediately after addition of labeled lipid nanoparticles; $I_{100\%}$ is the maximal fluorescence intensity value obtained after addition of detergent and I_t is the fluorescence intensity value measured at a given time *t*. Experiments were realized in triplicate.

Inhibition of GFP knockdown with Bafilomycin A1

In this experiment, HeLa/GFP cells were transfected with the CSL3-based lipid nanoparticles formulation (CSL3/DSPC/cholesterol/DSPE-PEG_{2000} 50:10:37.5:2.5 mol% - made by manual extrusion) in the presence or absence of Bafilomycin A1, a known inhibitor of vacuolar H^+ ATPases. HeLa/GFP cells were seeded in 12-well plates (40 000 cells/well) and allowed to attach overnight. The next day, cells were rinsed with Opti-MEM® and pre-incubated 30 minutes with Bafilomycin A1 (600 nM/well, diluted in Opti-MEM®) before incubation with lipid nanoparticles. Lipid nanoparticles and anti-GFP siRNA were complexed (N/P ratio of 4) as described above (for a final siRNA concentration of 10 and 25 nM/well). They were then diluted with Opti-MEM®, containing Bafilomycin A1 for a final concentration of 600 nM/well. Residual DMSO in the assay was 0.06% v/v (treated cells and negative control). Cells were then incubated for 4 hours with the lipid nanoparticles. After incubation, cells were rinsed twice with Opti-MEM® and 1 mL of complete culture media (EMEM/FBS 90:10 – with or without 600 nM Bafilomycin A1) was added. Cells were then incubated for 44 hours. After incubation, cells were rinsed with PBS, trypsinized and suspended in FACS buffer (95% PBS, 5% FBS, 1.0 mM EDTA) for immediate analysis *via* flow cytometry. GFP expression for each replicate (mean fluorescent intensity) was calculated relative to the control samples that did not receive any siRNA treatment. Experiments were realized in triplicate.

Comparison of the cellular uptake of the CSL3-based formulation in the presence or absence of Bafilomycin A1 over the 4-hour incubation period was assayed by flow cytometry. HeLa cells were seeded in 12-well plates (100 000 cells/well) and allowed to attach overnight. The next day, cells were rinsed with Opti-MEM® and pre-incubated 30 minutes with Bafilomycin A1 (600 nM/well, diluted in Opti-MEM®) before incubation with lipid nanoparticles. Lipid nanoparticles (25 µL in dextrose 5%) and siRNA-Alexa488 (25 µL in dextrose 5%) were complexed in dextrose 5% (N/P ratio of 4; siRNA concentration 25 nM/well) and were diluted with Opti-MEM®, containing Bafilomycin A1 for a final concentration of 600 nM/well. This solution was then transferred onto the cells (1300 µL final volume/well) and the plate was incubated for 4 hours. After incubation, cells were rinsed three times with EMEM/FBS 90:10, one time with PBS, trypsinized and suspended in FACS buffer (95% PBS, 5% FBS, 1.0 mM EDTA) for immediate analysis *via* flow cytometry. Experiments were realized in triplicate.

Uptake inhibition by flow cytometry

HeLa cells were incubated with siRNA-Alexa488 packaged with the CSL3-based lipid nanoparticles formulation (CSL3/DSPC/cholesterol/DSPE-PEG_{2000} 50:10:37.5:2.5 mol% - made by manual extrusion) in the presence or absence of endocytosis inhibitors. Inhibitors were used as follows: chlorpromazine (clathrin mediated endocytosis inhibitor) 10 µg/mL[9,10]; genistein (caveolae mediated endocytosis inhibitor) 200 µM[11]; Pitstop 2™ (clathrin and caveolae mediated endocytosis inhibitor) 20 µM[12]; EIPA (macropinocytosis inhibitor) 50 µM.[13] Discrimination of the clathrin and caveolae uptake pathways was not intended, because of the rather questionable specificity of the pharmacological endocytosis inhibitors between these two pathways.[12,14,15] Residual DMSO in the assay was 0.2% v/v (treated cells and negative control).

HeLa cells were seeded in 12-well plates (100 000 cells/well) and allowed to attach overnight. The next day, cells were rinsed with Opti-MEM® and pre-incubated 15 minutes with endocytosis inhibitors (diluted in Opti-MEM®) before incubation with lipid nanoparticles. Lipid nanoparticles and siRNA-Alexa488 were complexed in dextrose 5% (N/P ratio of 4; siRNA concentration 25 nM/well) and were diluted in Opti-MEM®. The solution was then transferred onto the cells (1300 µL final volume/well) and the plate was incubated for 1 or 6 hours. After incubation, cells were rinsed three times with Opti-MEM®/FBS 90:10, once with PBS, trypsinized and suspended in FACS buffer (95% PBS, 5% FBS, 1.0 mM EDTA) for immediate analysis *via* flow cytometry. Experiments were realized in triplicate.

Uptake inhibition was also confirmed by live-cell fluorescence microscopy. HeLa cells were routinely incubated 24 hours before imaging in 35 mm poly-d-lysine coated glass dishes (MatTek Corporation) at a density of 40 000 cells/dish. The next day, cells were rinsed with Opti-MEM® and pre-incubated 15 minutes with endocytosis inhibitors (diluted in Opti-MEM®) before incubation with lipid nanoparticles. siRNA-Alexa647 were complexed with lipid nanoparticles (N/P ratio of 4) as described previously (70 µL final volume). Complexes were then diluted with Opti-MEM® for a final siRNA concentration of 25 nM/dish. Cells were then incubated 1 hour with the lipid nanoparticles. After incubation, cells were rinsed twice with Opti-MEM®/FBS 90:10 and were stained with Hoechst 33342 (5 min, 2 µg/mL, Sigma). Cells were rinsed twice with Opti-MEM®/FBS 90:10 and were immediately imaged in a phenol red-free culture media (Opti-MEM®/FBS 90:10). Imaging of HeLa cells was performed using an Olympus IX81 fluorescent microscope equipped with a Plan Apo N 60X 1.42 NA silicone

objective (Olympus Canada Inc.) and a 12 bits Retiga-2000R CCD Camera (QImaging), using MetaMorph Advanced software 7.8.9 (Molecular Devices). Hoechst 33342 was imaged using the DAPI channel ($\lambda_{ex/em}$ 350/470) and siRNA-Alexa647 was imaged using the Cy5 channel ($\lambda_{ex/em}$ 649/666). All fluorescence images were carefully exported with constant scaling and range of greylevel. Experiments were realized in triplicate.

Lipid nanoparticle preparation by microfluidic mixing

Lipid nanoparticles were prepared according to previously reported procedures[16,17], using a Nanoassemblr™ microfluidic instrument with herringbone rapid mixing features (Precision Nanosystems, Vancouver, BC, Canada). Cationic switchable lipids, DSPC, cholesterol and DMG-PEG_{2000} were solubilized in ethanol at a molar ratio of 50:10:37.5:2.5. siRNA was diluted in 25 mM sodium acetate buffer pH 4.0. The siRNA/total lipid mass ratio was ~0.06, corresponding to a N/P ratio of 4. The two solutions were mixed at a flow rate of 12 mL/min, at a siRNA solution/lipid solution ratio of 3:1. Total volume of a batch is typically 2 mL (1.5 mL siRNA solution mixed with 0.5 mL of lipid solution). Nanoparticles were then dialyzed against saline (NaCl 0.9%) using Pur-A-Lyzer™ Maxi dialysis tubes MWCO 12-14 kDa (Sigma). If needed, nanoparticles were concentrated using Amicon Ultra-0.5 30K centrifugal filters device (EMD Millipore, Etobicoke, ON, Canada). Nanoparticles were then sterile filtered using a 0.2µm polyethersulfone 13 mm syringe filter (Pall Corporation, Mississauga, ON, Canada) and stored at 4°C until use. The siRNA encapsulation efficiency was quantified using a Ribogreen RNA quantitation assay (Thermo Scientific).[18] Nanoparticle size was measured by Dynamic Light Scattering in saline (0.9% NaCl) at 20°C as previously described.

The final siRNA concentration was determined *via* ion-pairing reversed phase liquid chromatography, according to a previously reported procedure.[19] siRNA were analyzed using a Waters XTerra® MS C_{18} column (2.1x50mm, 2.5 µm particle size) at 60°C on an Agilent 1100 HPLC system, with UV detection at 260 nm. Mobile phase A was 95% 0.1M triethylamine ammonium acetate (TEAA) pH 7.0 and 5% acetonitrile. Mobile phase B was 80% 0.1M TEAA pH 7.0 and 20% acetonitrile. The gradient changes from 0% to 70% mobile phase B for 12 min with a flow rate of 0.4 mL/min. Injection volume was 6 µL. Calibration curves of siRNA duplex usually ranges from 20 to 100 µg/mL. Before analysis, an aliquot of the lipid nanoparticle formulation was diluted with 200 mM TEAA pH 7.0 + 2% (v/v) $C_{12}E_8$ detergent to liberate encapsulated siRNA.

Biodistribution and ex-vivo imaging in mice

All procedures used in animal studies were approved by the Comité de Déontologie de l'Expérimentation sur les Animaux (CDEA – Animal Care and Ethical Committee) of the University of Montreal and were consistent with local, state and federal regulations. Mice were maintained at the animal facilities of the Institute for Research in Immunology and Cancer (IRIC) at the University of Montreal, and were housed under standard conditions. Food and water were provided *ad libitum*. Animals were acclimated to the animal facility for at least one week before experiments. Prior to injection, lipid nanoparticles formulations were diluted with saline (NaCl 0.9%) at siRNA concentrations such that each mouse was administered a dose of 10µL/g body-weight.

8-week-old male CD-1 mice (Charles River, Saint-Constant, QC, Canada) were injected intravenously *via* the tail vein with CSL3-based lipid nanoparticles (CSL3/DSPC/cholesterol/DMG-PEG_{2000} 50:10:37.5:2.5 mol% - made by microfluidic mixing) formulated with siRNA labeled with Cy5 on the sense strand, unformulated free siRNA-Cy5 or saline at a siRNA dose of 1.5 mg/kg. 4-hour post injection, mice were euthanized by CO_2 inhalation and organs (brain, lungs, heart, liver, spleen, kidneys) were immediately harvested and imaged using a Optix MX3 optical imaging system (Advanced Research Technologies, Montreal, QC, Canada). Data was processed using the OptiView® software from Advanced Research Technologies. Normalized photon counts (fluorescence intensity) was normalized by wet mass of organs. Experiments were realized in triplicate.

Serum stability study

This study was conducted as previously described by Zhou *et al*.[20] In order to estimate the stability of CSL3-based LNP (CSL3/DSPC/cholesterol/DMG-PEG_{2000} 50:10:37.5:2.5 mol% - made by microfluidic mixing) in the presence of plasma, DLS measurements of LNP/siRNA incubated into PBS, PBS/FBS 90:10 or PBS/FBS 50:50 (v/v) were realized using a Malvern Zetasizer Nano ZS (thermostated at 37°C, automatic algorithm mode). For the storage stability study in PBS, LNP were kept at +4°C between each measurement. Experiments were realized in triplicate.

In vivo Factor VII silencing in mice

Prior to injection, lipid nanoparticles formulations (CSL/DSPC/cholesterol/DMG-PEG_{2000} 50:10:37.5:2.5 mol% - made by microfluidic mixing) were diluted with saline (NaCl 0.9%) at siRNA concentrations such that each mouse was administered a dose of 10µL/g body-weight. 6 to 8-weeks old female C57BL/6 mice (Charles River, Saint-Constant, QC, Canada) received tail vein intravenous injection of saline (negative control, n=6) or LNP containing anti-Factor VII siRNA (n=4). After 48h, mice were euthanized and blood was collected *via* intracardiac sampling on citrate tubes. Serum was separated from whole blood using serum separation tubes (Becton Dickinson, Franklin Lakes, NJ, USA) and residual serum FVII levels were determined using the Biophen VII chromogenic assay (Aniara, West Chester, OH, USA) according to manufacturer's protocol. A standard curve was constructed using samples from saline-injected mice (pooled plasma, n=6) and relative Factor VII expression was determined by comparing treated groups to the standard curve.

References

1 W. Viricel, A. Mbarek and J. Leblond, *Angew. Chem. Int. Ed.*, 2015, **54**, 12743–12747.

2 M. Frank-Kamenetsky, A. Grefhorst, N. N. Anderson, T. S. Racie, B. Bramlage, A. Akinc, D. Butler, K. Charisse, R. Dorkin, Y. Fan, C. Gamba-Vitalo, P. Hadwiger, M. Jayaraman, M. John, K. N. Jayaprakash, M. Maier, L. Nechev, K. G. Rajeev, T. Read, I. Röhl, J. Soutschek, P. Tan, J. Wong, G. Wang, T. Zimmermann, A. de Fougerolles, H.-P. Vornlocher, R. Langer, D. G. Anderson, M. Manoharan, V. Koteliansky, J. D. Horton and K. Fitzgerald, *Proc. Natl. Acad. Sci. U. S. A.*, 2008, **105**, 11915–11920.

3 K. T. Love, K. P. Mahon, C. G. Levins, K. A. Whitehead, W. Querbes, J. R. Dorkin, J. Qin, W. Cantley, L. L. Qin, T. Racie, M. Frank-Kamenetsky, K. N. Yip, R. Alvarez, D. W. Y. Sah, A. de Fougerolles, K. Fitzgerald, V. Koteliansky, A. Akinc, R. Langer and D. G. Anderson, *Proc. Natl. Acad. Sci. U. S. A.*, 2010, **107**, 1864–1869.

4 D. A. Hay, F. M. Adam, G. Bish, F. Calo, R. Dixon, M. J. Fray, J. Hitchin, P. Jones, M. Paradowski, G. C. Parsons, K. J. W. Proctor, D. C. Pryde, N. N. Smith and T.-D. Tran, *Tetrahedron Lett.*, 2011, **52**, 5728–5732.

5 EP2781519 (A1), 2014.

6 C. Riemer, E. Borroni, B. Levet-Trafit, J. R. Martin, S. Poli, R. H. P. Porter and M. Bös, *J. Med. Chem.*, 2003, **46**, 1273–1276.

7 S. Poirier, M. Mamarbachi, W.-T. Chen, A. S. Lee and G. Mayer, *Cell Rep.*, 2015, **13**, 2064–2071.

8 L. Yao, J. Daniels, D. Wijesinghe, O. A. Andreev and Y. K. Reshetnyak, *J. Controlled Release*, 2013, **167**, 228–237.

9 T. dos Santos, J. Varela, I. Lynch, A. Salvati and K. A. Dawson, *PLoS ONE*, 2011, **6**, e24438.

10 K. von Gersdorff, N. N. Sanders, R. Vandenbroucke, S. C. De Smedt, E. Wagner and M. Ogris, *Mol. Ther.*, 2006, **14**, 745–753.

11 J. Rejman, A. Bragonzi and M. Conese, *Mol. Ther.*, 2005, **12**, 468–474.

12 D. Dutta, C. D. Williamson, N. B. Cole and J. G. Donaldson, *PLoS ONE*, 2012, **7**, e45799.

13 J. Gilleron, W. Querbes, A. Zeigerer, A. Borodovsky, G. Marsico, U. Schubert, K. Manygoats, S. Seifert, C. Andree, M. Stöter, H. Epstein-Barash, L. Zhang, V. Koteliansky, K. Fitzgerald, E. Fava, M. Bickle, Y. Kalaidzidis, A. Akinc, M. Maier and M. Zerial, *Nat. Biotechnol.*, 2013, **31**, 638–646.

14 A. K. Willox, Y. M. E. Sahraoui and S. J. Royle, *Biol. Open*, 2014, BIO20147955.

15 D. Vercauteren, R. E. Vandenbroucke, A. T. Jones, J. Rejman, J. Demeester, S. C. De Smedt, N. N. Sanders and K. Braeckmans, *Mol. Ther.*, 2010, **18**, 561–569.

16 B. L. Mui, Y. K. Tam, M. Jayaraman, S. M. Ansell, X. Du, Y. Y. C. Tam, P. J. Lin, S. Chen, J. K. Narayanannair, K. G. Rajeev, M. Manoharan, A. Akinc, M. A. Maier, P. Cullis, T. D. Madden and M. J. Hope, *Mol. Ther. — Nucleic Acids*, 2013, **2**, e139.

17 Y. Zhang, J. M. Pelet, D. A. Heller, Y. Dong, D. Chen, Z. Gu, B. J. Joseph, J. Wallas and D. G. Anderson, *Adv. Mater.*, 2013, **25**, 4641–4645.

18 C. Walsh, K. Ou, N. Belliveau, T. Leaver, A. Wild, J. Huft, P. Lin, S. Chen, A. Leung, J. Lee, C. Hansen, R. Taylor, E. Ramsay and P. Cullis, in *Drug Delivery System*, ed. K. K. Jain, Springer New York, 2014, pp. 109–120.

19 V. Murugaiah, W. Zedalis, G. Lavine, K. Charisse and M. Manoharan, *Anal. Biochem.*, 2010, **401**, 61–67.

20 K. Zhou, L. H. Nguyen, J. B. Miller, Y. Yan, P. Kos, H. Xiong, L. Li, J. Hao, J. T. Minnig, H. Zhu and D. J. Siegwart, *Proc. Natl. Acad. Sci.*, 2016, **113**, 520–525.