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Electronic Supplementary Information

Synthesis and Characterization of Novel Zwitterionic Lipids with pH-Responsive Biophysical Properties

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Materials and Methods

Chemical synthesis, characterization, and purification

1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC), 1,2-stearoyl-*sn*-glycero-3-phosphocholine (DSPC), 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE), 1,2-dioleoyl-*sn*-glycero-3-phospho-(1'-rac-glycerol) (DOPG), 1,2-dioleoyl-3-trimethylamonium-propane (DOTAP), and cholesterol (Chol) were purchased from Avanti Polar Lipids (Alabaster, AL). t-butyl p-(bromomethyl) benzoate was purchased from Cayman Chemical (Ann Arbor, MI). All other reagents were purchased from Sigma-Aldrich (Milwaukee, WI). Solvents were purchased from VWR (Radnor, PA), and used without further purification. TLC analyses were performed on 0.25 mm silica gel F_{254} plates using the described solvent systems. High-performance flash chromatography (HPFC) was performed on a Grace (Deerfield, IL) Reveleris HPFC system with pre-packed Reveleris silica gel columns (70 Å, 40 μm). ^{1}H NMR spectra were acquired using a Bruker 300 Avance MHz instrument. Chemical shifts are expressed as parts per million, and tetramethylsilane was used as an internal standard. MALDI-TOF spectra were acquired using an Applied Biosystems (Foster City, CA) Voyager-DE workstation. The detailed synthetic schemes are described below.

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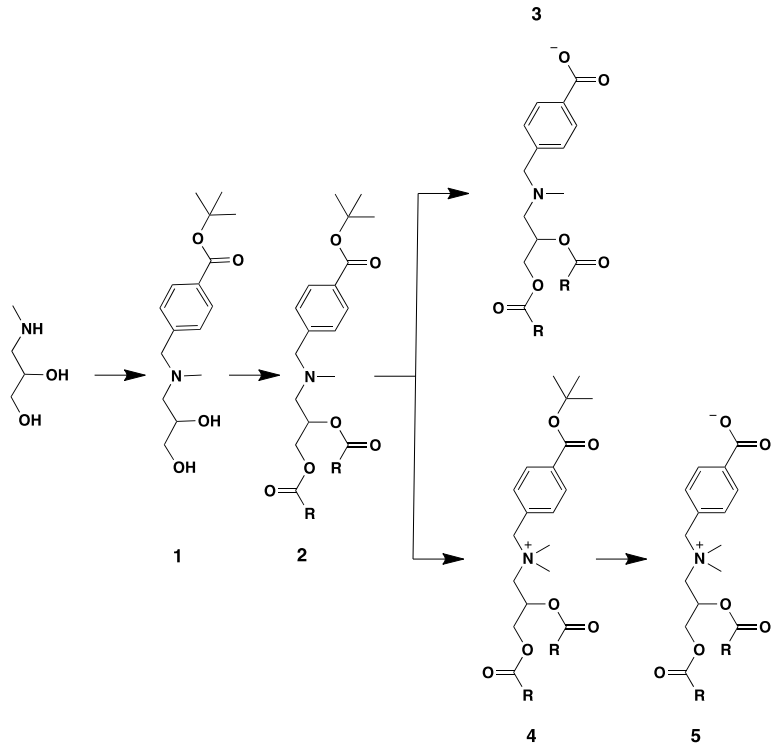
Synthesis of 4-(((2,3-bis(oleoyloxy)propyl)(methyl)amino)methyl)benzoic acid (DOBAT) and *N*-(4-carboxybenzyl)-*N*,*N*-dimethyl-2,3-bis(oleoyloxy)propan-1-aminium (DOBAQ)



Scheme 1: DOBAT and DOBAQ Synthesis

0.6 g (18 mmol) K_2CO_3 was suspended in 35 ml of a 1:1 methanol:THF solution. To the suspension was added 1g (3.7 mmol) t-butyl-p-bromobenzoate and 0.6 g (5.5 mmol) 3-methylamino-1,2-propanediol. The mixture was stirred at 35 °C for 16 hrs. The solid K_2CO_3 was filtered off, and the resulting mixture was concentrated in vacuo, resuspended in minimal DCM, and purified by HPFC using a 0-10% methanol in chloroform gradient, which provided 0.65 g (60% yield) of **1** as clear oil. TLC: R_f = 0.5 (eluent 9:1 $CHCl_3$:MeOH). ^{1}H NMR ($CDCl_3$): δ 1.55 (s, 9H); δ 2.40 (s, 3H) δ 2.60 (m, 2H); δ 3.55 (m, 2H); δ 3.70 (m, 2H); δ 3.90 (m, 1H); δ 7.40 (m, 2H); δ 7.90 (m, 2H). MALDI-MS $[M+H]^+$ - calculated 295.18, found 295.8.

A mixture of 0.6 g (2.2 mmol) **1**, 1.3 g (4.8 mmol) oleic acid, 1.05 g (5.5 mmol) DCC, and 0.27 g (2.2 mmol) DMAP in dry DCM (25 ml) was stirred at room temperature for 16 hr. Precipitated DCU salt was removed by filtration. An additional 75 ml of DCM was added, and the organic phase was washed with 1M HCl (2 x 25 ml), water (2 x 25 ml), and brine (30 ml). The organic was then dried over anhydrous Na_2SO_4 and concentrated by evaporation under reduced pressure to yield a yellow oil as the crude

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product. This oil was further purified by HPFC using a 0-4% methanol in chloroform gradient to yield 1.21 g (72% yield) of **2** as a clear oil. TLC: R_f = 0.4 (99:1 $CHCl_3$:MeOH). ^{1}H NMR ($CDCl_3$): δ 0.90 (t, 6H); δ 1.20-1.50 (m, 40H); δ 1.55 (s, 9H); δ 1.62 (m, 4H); δ 2.04 (m, 8H); δ 2.30 (m, 4H); δ 2.40 (s, 3H); δ 2.75 (m, 2H); δ 3.55 (m, 2H); δ 4.25 (m, 2H); δ 5.30 (m, 4H); δ 5.40 (m, 1H); δ 7.40 (m, 2H); δ 7.90 (m, 2H). MALDI-MS $[M+H]^+$ - calculated 823.67, found 824.3.

DOBAT synthesis

0.5 g (0.6 mmol) of **2** was stirred for 1 hr at room temperature in a solution of 5:4:1 DCM:TFA:TIPS (10 ml). Solvent was removed by evaporation at reduced pressure. The product was purified by HPFC using an elution gradient of 0-10% methanol in chloroform, giving 0.44 g (95% yield) of final product **3**. TLC: R_f = 0.1 (99:1 $CHCl_3$:MeOH). ^{1}H NMR ($CDCl_3$): δ 0.90 (t, 6H); δ 1.20-1.50 (m, 40H); δ 1.62 (m, 4H); δ 2.04 (m, 8H); δ 2.30 (m, 4H); δ 2.40 (s, 3H); δ 2.75 (m, 2H); δ 3.55 (m, 2H); δ 4.25 (m, 2H); δ 5.30 (m, 4H); δ 5.40 (m, 1H); δ 7.40 (m, 2H); δ 7.90 (m, 2H). MALDI-MS $[M+H]^+$ - calculated 768.61, found 768.2.

DOBAQ synthesis

To a solution of 0.85 g (1 mmol) **2** dissolved in 10 ml acetone stirring at 0 °C, 0.5 ml (5 mmol) was added dropwise, and the reaction was allowed to slowly warm to room temperature, then stirred at room temperature for 16 hr. Solvent was removed by evaporation in vacuo, the crude oil was solubilized in 50 ml DCM, washed with water (2 x 15 ml) and brine (20 ml), and dried over Na_2SO_4. The crude product was purified by HPLC using a 0-20% methanol in chloroform gradient to yield 0.52 g (60% yield) of **4**. TLC: R_f = 0.2 (95:5 $CHCl_3$:MeOH). ^{1}H NMR ($CDCl_3$): δ 0.90 (t, 6H); δ 1.20-1.50 (m, 40H); δ 1.55 (s, 9H); δ 1.62 (m, 4H); δ 2.04 (m, 8H); δ 2.30 (m, 4H); δ 3.20 (s, 3H); δ 3.3 (s, 3H); δ 3.90 (m, 2H); δ 4.30 (m, 2H); δ 4.80 (m, 2H); δ 5.30 (m, 4H); δ 5.50 (m, 1H); δ 7.70 (m, 2H); δ 8.10 (m, 2H). MALDI-MS $[M+H]^+$ - calculated 838.69, found 839.7.

0.5 g (0.6 mmol) of **4** was stirred for 1 hr at room temperature in a solution of 5:4:1 DCM:TFA:TIPS (10 ml). Solvent was removed by evaporation at reduced pressure. The product was purified by HPFC using an elution gradient of 0-30% methanol in chloroform, giving 0.41 g (87% yield) of final product **5**. TLC: R_f = 0.75 (65:25:4 $CHCl_3$:MeOH:NH_3OH). ^{1}H NMR ($CDCl_3$): δ 0.90 (t, 6H); δ 1.20-1.50 (m, 40H); δ 1.62 (m, 4H); δ 2.04 (m, 8H); δ 2.30 (m, 4H); δ 3.20 (s, 3H); δ 3.3 (s, 3H); δ 3.90 (m, 2H); δ 4.30 (m, 2H); δ 4.80 (m, 2H); δ 5.30 (m, 4H); δ 5.60 (m, 1H); δ 7.70 (m, 2H); δ 8.10 (m, 2H). MALDI-MS $[M+H]^+$ - calculated 782.63, found 783.2.

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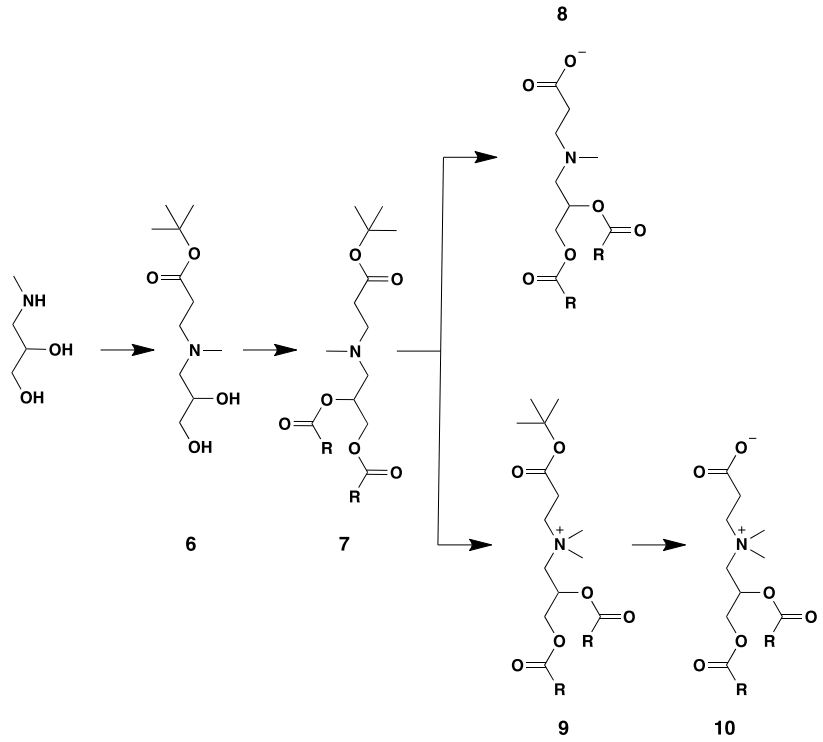
Synthesis of 3-((2,3-bis(oleoyloxy)propyl)(methyl)amino)propanoic acid (DOPAT) and *N*-(2-carboxyethyl)-*N*,*N*-dimethyl-2,3-bis(oleoyloxy)propan-1-aminium (DOPAQ)



Scheme 2: DOPAT and DOPAQ Synthesis

1 g (9.5 mmol) of 3-methylamino-1,2-propanediol and 1.34 g (10.5 mmol) t-butyl acrylate were stirred neat at 75 °C for 16 hr. Product (**6**) was moved forward without purification due to nearly quantitative yield (2.30 g). TLC: R_f = 0.66 (90:10:1 $CHCl_3$:MeOH:NH_3OH). ^1H NMR ($CDCl_3$): δ 1.50 (s, 9H); δ 2.30 (s, 3H); δ 2.40 (m, 2H); δ 2.65 (m, 2H); δ 2.85 (m, 2H); δ 3.5 (m, 1H); δ 3.8 (m, 2H). MALDI-MS $[M+H]^+$ - calculated 233.16, found 233.8.

A mixture of 0.8 g (3.4 mmol) **6**, 2.1 g (7.5 mmol) oleic acid, 1.75 g (8.5 mmol) DCC, and 0.42 g (3.4 mmol) DMAP in dry DCM (35 ml) was stirred at room temperature for 16 hr. Precipitated DCU salt was removed by filtration. An additional 75 ml of DCM was added, and the organic phase was washed with 1M HCl (2 x 25 ml), water (2 x 25 ml), and brine (30 ml). The organic was then dried over anhydrous Na_2SO_4 and concentrated by evaporation under reduced pressure to yield a yellow oil as the crude product. This oil was further purified by HPFC using a 0-5% methanol in chloroform gradient to yield 2.1 g (80% yield) of **7** as a clear oil. TLC: R_f = 0.66 (95:5 $CHCl_3$:MeOH). ^1H NMR ($CDCl_3$): δ 0. 90 (t, 6H); δ 1.20-1.50 (m, 40H); δ 1.50 (s, 9H);

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δ 1.65 (m, 4H); δ 2.04 (m, 8H); δ 2.30 (m, 4H); δ 2.40 (s, 3H); δ 2.45 (m, 2H); δ 2.50 (m, 2H); δ 2.80 (m, 2H); δ 4.30 (m, 2H); δ 5.30 (m, 4H); δ 5.40 (m, 1H). MALDI-MS $[M+H]^+$ - calculated 761.65, found 762.8.

DOPAT synthesis

0.75 g (1 mmol) of **7** was stirred for 1 hr at room temperature in a solution of 5:4:1 DCM:TFA:TIPS (10 ml). Solvent was removed by evaporation at reduced pressure. The product was purified by HPFC using an elution gradient of 0-10% methanol in chloroform, giving 0.65 g (93% yield) of final product **8**. TLC: R_f = 0.1 (95:5 $CHCl_3$:MeOH). ^{1}H NMR ($CDCl_3$): δ 0. 90 (t, 6H); δ 1.20-1.50 (m, 40H); δ 1.65 (m, 4H); δ 2.04 (m, 8H); δ 2.30 (m, 4H); δ 2.40 (s, 3H); δ 2.45 (m, 2H); δ 2.50 (m, 2H); δ 2.80 (m, 2H); δ 4.30 (m, 2H); δ 5.30 (m, 4H); δ 5.40 (m, 1H). MALDI-MS $[M+H]^+$ - calculated 706.6, found 706.4.

DOPAQ synthesis

To a solution of 1.2 g (1.6 mmol) **7** dissolved in 15 ml acetone stirring at 0 °C, 1 ml (10 mmol) was added dropwise, and the reaction was allowed to slowly warm to room temperature, then stirred at room temperature for 16 hr. Solvent was removed by evaporation in vacuo, the crude oil was solubilized in 100 ml DCM, washed with water (2 x 20 ml) and brine (25 ml), and dried over Na_2SO_4. The crude product was purified by HPLC using a 0-20% methanol in chloroform gradient to yield 0.7 g (57% yield) of **9**. TLC: R_f = 0.1 (95:5 $CHCl_3$:MeOH). ^{1}H NMR ($CDCl_3$): δ 0. 90 (t, 6H); δ 1.20-1.50 (m, 40H); δ 1.50 (s, 9H); δ 1.65 (m, 4H); δ 2.04 (m, 8H); δ 2.30 (m, 4H); δ 3.10 (m, 2H); δ 3.20 (s, 3H); δ 3.30 (s, 3H); δ 3.75 (m, 2H); δ 3.95 (m, 2H); δ 4.30 (m, 2H); δ 5.30 (m, 4H); δ 5.40 (m, 1H). MALDI-MS $[M+H]^+$ - calculated 776.68, found 777.8.

0.7 g (0.9 mmol) of **9** was stirred for 1 hr at room temperature in a solution of 5:4:1 DCM:TFA:TIPS (10 ml). Solvent was removed by evaporation at reduced pressure. The product was purified by HPFC using an elution gradient of 0-25% methanol in chloroform, giving 0.5 g (77% yield) of final product **10**. TLC: R_f = 0.5 (80:20 $CHCl_3$:MeOH). ^{1}H NMR ($CDCl_3$): δ 0. 90 (t, 6H); δ 1.20-1.50 (m, 40H); δ 1.65 (m, 4H); δ 2.04 (m, 8H); δ 2.30 (m, 4H); δ 3.10 (m, 2H); δ 3.20 (s, 3H); δ 3.30 (s, 3H); δ 3.75 (m, 2H); δ 3.95 (m, 2H); δ 4.30 (m, 2H); δ 5.30 (m, 4H); δ 5.60 (m, 1H). MALDI-MS $[M+H]^+$ - calculated 720.61, found 721.6.

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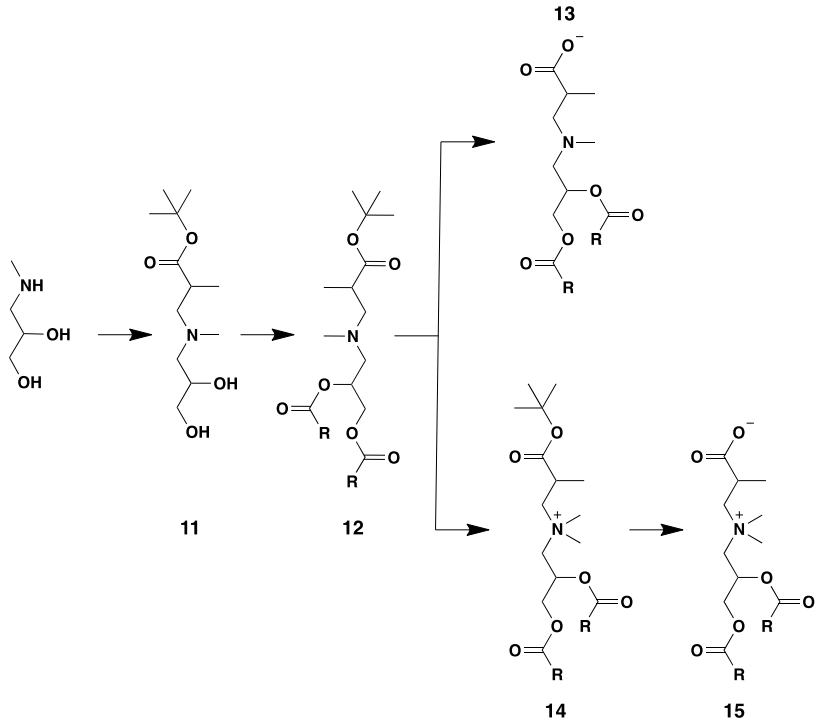
Synthesis of 3-((2,3-bis(oleoyloxy)propyl)(methyl)amino)-2-methylpropanoic acid (DOMPAT) and *N*-(2-carboxypropyl)-*N*,*N*-dimethyl-2,3-bis(oleoyloxy)propan-1-aminium (DOMPAQ)



Scheme 3: DOMPAT and DOMPAQ Synthesis

1 g (9.5 mmol) of 3-methylamino-1,2-propanediol and 1.49 g (10.5 mmol) t-butyl methacrylate were stirred neat at 75 °C for 16 hr. Product (**11**) was moved forward without purification due to nearly quantitative yield (2.45 g). TLC: R_f = 0.7 (90:10:1 $CHCl_3$:MeOH:NH_3OH). ^1H NMR ($CDCl_3$): δ 1.10 (m, 3H); δ 1.50 (s, 9H); δ 2.30 (s, 3H); δ 2.35 (m, 2H); δ 2.40 (m, 1H); δ 2.45 (m, 2H); δ 2.65 (m, 2H); δ 3.75 (m, 1H). MALDI-MS $[M+H]^+$ - calculated 247.18, found 248.4.

A mixture of 1.5 g (6.1 mmol) **11**, 3.77 g (13.4 mmol) oleic acid, 3.1 g (15 mmol) DCC, and 0.74 g (6.1 mmol) DMAP in dry DCM (60 ml) was stirred at room temperature for 16 hr. Precipitated DCU salt was removed by filtration. An additional 75 ml of DCM was added, and the organic phase was washed with 1M HCl (2 x 25 ml), water (2 x 25 ml), and brine (30 ml). The organic was then dried over anhydrous Na_2SO_4 and concentrated by evaporation under reduced pressure to yield a yellow oil as the crude product. This oil was further purified by HPFC using a 0-5% methanol in chloroform gradient to yield 3.6 g (77% yield) of **12** as a clear oil. TLC: R_f = 0.7 (95:5

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$CHCl_3$:MeOH). ^1H NMR ($CDCl_3$): δ 0.90 (t, 3H); δ 1.10 (m, 3H); δ 1.20-1.50 (m, 40H); δ 1.50 (s, 9H); δ 1.65 (m, 4H); δ 2.04 (m, 8H); δ 2.30 (m, 2H); δ 2.32 (s, 3H); δ 2.35 (m, 4H); δ 2.40 (m, 1H); δ 2.80 (m, 2H); δ 4.30 (m, 2H); δ 5.30 (m, 4H); δ 5.40 (m, 1H). MALDI-MS $[M+H]^+$ - calculated 775.67, found 777.4.

DOMPAT synthesis

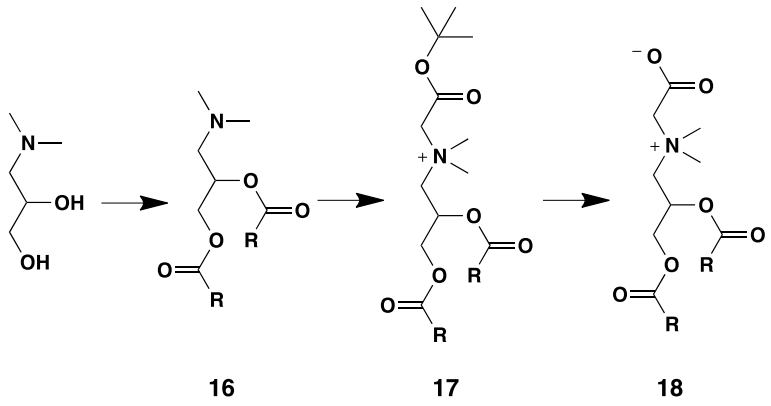
1.5 g (1.9 mmol) of **12** was stirred for 1 hr at room temperature in a solution of 5:4:1 DCM:TFA:TIPS (20 ml). Solvent was removed by evaporation at reduced pressure. The product was purified by HPFC using an elution gradient of 0-10% methanol in chloroform, giving 1.24 g (90% yield) of final product **13**. TLC: R_f = 0.15 (95:5 $CHCl_3$:MeOH). ^1H NMR ($CDCl_3$): δ 0.90 (t, 3H); δ 1.10 (m, 3H); δ 1.20-1.50 (m, 40H); δ 1.65 (m, 4H); δ 2.04 (m, 8H); δ 2.30 (m, 2H); δ 2.32 (s, 3H); δ 2.35 (m, 4H); δ 2.40 (m, 1H); δ 2.80 (m, 2H); δ 4.30 (m, 2H); δ 5.30 (m, 4H); δ 5.40 (m, 1H). MALDI-MS $[M+H]^+$ - calculated 720.61, found 722.8.

DOMPAQ synthesis

To a solution of 1 g (1.3 mmol) **12** dissolved in 15 ml acetone stirring at 0 °C, 1 ml (10 mmol) was added dropwise, and the reaction was allowed to slowly warm to room temperature, then stirred at room temperature for 16 hr. Solvent was removed by evaporation in vacuo, the crude oil was solubilized in 100 ml DCM, washed with water (2 x 20 ml) and brine (25 ml), and dried over Na_2SO_4. The crude product was purified by HPLC using a 0-20% methanol in chloroform gradient to yield 0.61 g (59% yield) of **14**. TLC: R_f = 0.2 (90:10:1 $CHCl_3$:MeOH:NH_3OH). ^1H NMR ($CDCl_3$): δ 0.90 (t, 3H); δ 1.20-1.50 (m, 40H); δ 1.30 (m, 3H); δ 1.50 (s, 9H); δ 1.65 (m, 4H); δ 2.04 (m, 8H); δ 2.35 (m, 4H); δ 3.15 (m, 1H); δ 3.20 (s, 3H); δ 3.30 (s, 3H); δ 3.80 (m, 2H); δ 3.90 (m, 2H); δ 4.30 (m, 2H); δ 5.30 (m, 4H); δ 5.40 (m, 1H). MALDI-MS $[M+H]^+$ - calculated 790.69, found 792.8.

0.61 g (0.8 mmol) of **14** was stirred for 1 hr at room temperature in a solution of 5:4:1 DCM:TFA:TIPS (10 ml). Solvent was removed by evaporation at reduced pressure. The product was purified by HPFC using an elution gradient of 0-25% methanol in chloroform, giving 0.44 g (74% yield) of final product **15**. TLC: R_f = 0.2 (90:10:1 $CHCl_3$:MeOH:NH_3OH). ^1H NMR ($CDCl_3$): δ 0.90 (t, 3H); δ 1.20-1.50 (m, 40H); δ 1.30 (m, 3H); δ 1.65 (m, 4H); δ 2.04 (m, 8H); δ 2.35 (m, 4H); δ 3.15 (m, 1H); δ 3.20 (s, 3H); δ 3.30 (s, 3H); δ 3.80 (m, 2H); δ 3.90 (m, 2H); δ 4.30 (m, 2H); δ 5.30 (m, 4H); δ 5.40 (m, 1H). MALDI-MS $[M+H]^+$ - calculated 734.63, found 736.7.

Synthesis of *N*-(carboxymethyl)-*N*,*N*-dimethyl-2,3-bis(oleoyloxy)propan-1-aminium (DOAAQ)



Scheme 4: DOAAQ Synthesis

A mixture of 0.5 g (4.2 mmol) 3-(dimethylamino)-1,2-propanediol, 2.61 g (9.25 mmol) oleic acid, 2.2 g (10.5 mmol) DCC, and 0.51 g (4.2 mmol) DMAP in dry DCM (40 ml) was stirred at room temperature for 16 hr. Precipitated DCU salt was removed by filtration. An additional 60 ml of DCM was added, and the organic phase was washed with 1M HCl (2 x 25 ml), water (2 x 25 ml), and brine (30 ml). The organic was then dried over anhydrous Na_2SO_4 and concentrated by evaporation under reduced pressure to yield a yellow oil as the crude product. The crude product was purified by HPFC using an elution gradient of 0-5% methanol in chloroform, giving 2.47 g (91% yield) of **16** as a yellow oil. TLC: R_f = 0.7 (95:5 $CHCl_3$:MeOH). ^{1}H NMR ($CDCl_3$): δ 0.90 (t, 3H); δ 1.20-1.50 (m, 40H); δ 1.65 (m, 4H); δ 2.04 (m, 8H); δ 2.30 (s, 6H); δ 2.35 (m, 4H); δ 2.60 (m, 2H); δ 4.30 (m, 2H); δ 5.30 (m, 4H); δ 5.60 (m, 1H). MALDI-MS $[M+H]^+$ - calculated 647.59, found 649.8.

1.5 g (2.3 mmol) of **16** in THF (30 ml) was stirred at 40 °C with 0.7 g (3.45 mmol) t-butyl bromoacetate and 0.6 g (4.6 mmol) Hunig's base for 48 hrs. Solvent was removed by evaporation under reduced pressure, and the crude oil was resuspended in 50 ml DCM and washed with water (2 X 15 ml) and brine (15 ml). The organic was dried over anhydrous Na2SO4 and concentrated by evaporation under reduced pressure to yield a yellow oil as the crude product. The product was purified by HPFC using an elution gradient of 0-10% methanol in chloroform, giving 0.8 g (45% yield) of **17** as a yellow oil. TLC: R_f = 0.15 (95:5 $CHCl_3$:MeOH). ^{1}H NMR ($CDCl_3$): δ 0.90 (t, 3H); δ 1.20-1.50 (m, 40H); δ 1.50 (s, 9H); δ 1.65 (m, 4H); δ 2.04 (m, 8H); δ 2.35 (m, 4H); δ 3.50 (s, 3H); δ 3.60 (s, 3H); δ 3.90 (m, 2H); δ 4.30 (m, 2H); δ 4.35 (m, 2H); δ 5.30 (m, 4H); δ 5.60 (m, 1H). MALDI-MS $[M+H]^+$ - calculated 762.66, found 763.2.

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0.8 g (1 mmol) of **17** was stirred for 1 hr at room temperature in a solution of 5:4:1 DCM:TFA:TIPS (20 ml). Solvent was removed by evaporation at reduced pressure. The product was purified by HPFC using an elution gradient of 0-20% methanol in chloroform, giving 0.65 g (88% yield) of final product **18**. TLC: R_f = 0.1 (90:10:1 $CHCl_3$:MeOH:NH_3OH). ^1H NMR ($CDCl_3$): δ 0.90 (t, 3H); δ 1.20-1.50 (m, 40H); δ 1.65 (m, 4H); δ 2.04 (m, 8H); δ 2.35 (m, 4H); δ 3.50 (s, 3H); δ 3.60 (s, 3H); δ 3.90 (m, 2H); δ 4.30 (m, 2H); δ 4.35 (m, 2H); δ 5.30 (m, 4H); δ 5.60 (m, 1H). MALDI-MS $[M+H]^+$ - calculated 706.60, found 708.4.

Buffers

Unless otherwise stated, isosmotic buffers containing 10 mM buffering agent and 50 mM NaCl were used for all experiments. Buffers from pH=3.0-8.5 in 0.5 pH increments were used. The buffering agent used was dependent on the desired pH of the buffer. Sodium acetate was used for pH=3.0-5.5; 2-(*N*-morpholino)ethanesulfonic acid (MES) was used for pH=6.0-7.0; tris(hydroxymethyl)aminomethane (TRIS-HCl) was used for pH=7.5-8.5.

Size and zeta zotential measurements

The mean diameter of liposomes for all experiments was determined by dynamic light scattering using a Zetasizer NanoZS (Malvern, Westborough MA). 10 μL of liposome solution (10 mM) was diluted into 740 μL of 10 mM Tris-HCl, 50 mM NaCl (pH=7.4) for size measurements.

Zeta potential measurements of liposomal formulations containing betaine-like lipids were performed at various pH values using a Zetasizer NanoZS (Malvern, Westborough MA). 10 μL of 10 mM liposomal solution was diluted into 740 μl of isosmotic buffer in a dip cell cuvette for zeta potential measurements

Zeta potential of ZL:DOPC formulations

Dry lipid films of the desired ZL:DOPC lipid ratios (5 μmol total lipid) were hydrated in 0.5 ml of buffer (pH=8.5), sonicated two minutes to disperse the lipid film, then extruded 11 times through a 200 nm polycarbonate membrane (Whatman International, Kent, UK) using a handheld extruder (Avestin, Ottawa, ON, Canada) extruder at room temperature. Zeta potential was measured as a function of pH from pH=3.0-8.5 in 0.5 pH intervals. Measurements were taken four times per sample and averaged to give the final value and standard deviation.

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Determination of pH-dependent membrane aggregation of liposomes through a FRET based lipid mixing assay

Dry lipid films 43:30:25:1:1 DOPC:ZL:DOPE:Rho-PE:NBD-PE were rehydrated at a lipid concentration of 10 mM in buffer (pH=8.5), sonicated 2 minutes to disperse the lipid film, then extruded 11 times through 100 nm polycarbonate membrane at room temperature. Dry lipid films containing 45:20:20:15 DOPC:DOPE:DOPG:Chol (acceptor composition) were rehydrated at a lipid concentration of 10 mM in buffer (pH=8.5), sonicated two minutes to disperse the lipid film, then extruded 11 times through 100 nm polycarbonate membrane at room temperature.

Lipid mixing between ZL containing liposomes and anionic liposomes was investigated using fluorescence resonance energy transfer (FRET) to determine the fusogenic potential of ZL.[1] The DOPE-conjugated FRET probes lissamine rhodamine B (Rho-PE) and 7-nitrobenzo-2-oxa-1,3-diazole (NBD-PE) were combined into a single liposomal formulation, resulting in attenuated NBD fluorescence due to FRET with Rho. Upon lipid mixing with a probe-free acceptor vesicle, NBD signal increases due to an increase in average distance between the two probes as they redistribute across the two membranes. For mixing experiments, 2 μL of donor vesicles (10 mM) were added to 1980 μL of buffer and the baseline fluorescence was measured (F_{min}). 18 μL of acceptor liposomes (10 mM) were injected, and fluorescence was measured after 5 minutes at 37 °C (F). 30 μL of 15% $C_{12}E_{10}$ were then added, and the fluorescence was measured after 30 seconds (F_{max}). % lipid mixing was defined as $(F-F_{min})/(F_{max}-F_{min})*100$, and was reported as the average of triplicate measurements. All fluorescence measurements were made on a Spex Fluorolog fluorimeter (Horiba Jobin Yvon, Edison NJ) at Ex/Em = 465/520 nm.

Lysis of biomembrane mimicking vesicles

Dry lipid films of anionic biomembrane mimicking vesicles (BMV) containing 45:20:20:15 DOPC:DOPE:DOPG:Chol[2] were hydrated at a lipid concentration of 10 mM in a solution containing 12.5 mM ANTS, 42.5 mM DPX, 10 mM Tris-HCl, 20 mM NaCl (pH=7.4), and sonicated 10 minutes at 25 °C to form a homogeneous dispersion.[3] Unencapsulated ANTS/DPX was removed by column purification on a Sephadex G-25 (PD-10) desalting column using an isosmotic buffer (pH=7.4) to a final concentration of 5 mM. Dry lipid films of ZL vesicles containing 45:30:25 DOPE:ZL:DOPC were hydrated at a lipid concentration of 5 mM in buffer containing 10 mM Tris-HCl and 150 mM NaCl (pH= 7.4), sonicated 2 minutes to disperse the lipid film, then extruded 11 times through 100 nm polycarbonate membrane at room temperature.

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10 µL of purified ANTS/DPX containing liposomes diluted into 1 ml of buffer (final concentration of 50 µM) containing 10 mM Tris-HCl and 150 mM NaCl (pH=7.4) or 10 mM sodium acetate and 150 mM NaCl (pH=4.0). 20 µL of ZL containing vesicles were added (final concentration 100 µM), and the ANTS fluorescence (Ex/Em = 360nm/530nm) was measured at t = 0 min (F_0) and t = 30 min (F_{30}). 5 µL of a 15% $C_{12}E_{10}$ solution was added to lyse all vesicles, and the max fluorescence (F_{max}) was measured. The percentage of free ANTS at t = 30 min should correlate with membrane lysis, and is reported as % lysis = $(F_{30}-F_0)/(F_{max}-F_0)*100$. The data reported are the average of triplicate measurements, and all fluorescence measurements were made on a Spex Fluorolog fluorimeter (Horiba Jobin Yvon, Edison NJ).

Encapsulation of siRNA in ZL liposomes

ZL lipid nanoparticles were prepared using a batch mixing process at 25°C. 2 mg total lipid was dissolved in 250 µL of ethanol and sonicated at 25°C for 5 min. The lipid/ethanol solution was subsequently injected into a magnetically stirred 2.5 ml vial, which contained 100 µg of siRNA dissolved in 250 µL of 50 mM citric acid buffer, pH 4. For reproducible injection speed, a KD Scientific pump holding a syringe with a 22 G x 1 1/2 needle was used to inject the ethanol lipid solution into the aqueous phase. The injection rate was 5 mL/min. The lipid suspension was stirred for 10 min and then extruded through 80 nm polycarbonate membranes 5 times at room temperature. Ethanol was removed by dialysis against PBS (pH 7.4) without Ca^{2+} and Mg^{2+} for 24h. Encaspulation efficiency of siRNA was quantified by measuring the fluorescence signal upon addition of Ribogreen to aliquots of lipid nanoparticle formulations in the presence or absence of 0.4% Triton-X. Fluorescence was measured using the Fluostar fluorescence plate reader (BMG Labtech, Cary, NC) (Ex/Em = 485/520 nm). siRNA was quantified by a calibration curve ranging from 50 ng/ml to 1µg/ml siRNA. Liposome size and zeta potential were measured 3 times per sample on a Zetasizer NanoZS (Malvern, Westborough MA) and averaged.

In vitro knockdown in Hela-Luc cells

Stably transfected HeLa-Luc cells were cultured in MEM Eagles's with Earle's BSS Medium supplemented with 10% heat-inactivated FCS. Twenty-four hours before transfection, cells were seeded in 96-well plates at a density of 8,000/well. Liposomal formulations (65:35:1 DOBAQ:DOPE:PEG-DMG) correlating to 90 or 180 nM siRNA were added to cells in medium supplemented with 10% fetal calf serum. Weight/weight (ZL/siRNA) ratios of 15 and 30 were used. After 24 h incubation, medium was replaced, and the cells were analyzed for luciferase gene silencing. In addition to anti-luc siRNA, non-specific siRNA was used to assay for gene silencing by cytotoxic or off-target effects. Luciferase gene silencing activity was measured according to the protocol provided by

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Promega (Madison, WI, USA). Briefly, luciferase light units were quantified by adding 100 µL of Steady-Glo (Promega, Madison, WI, USA) to the cells containing 100 µL of media per well. The relative light units (RLU) were measured with a 1450 MicroBeta Trilux, Liquid Scintillation and Luminescence Counter (Perkin Elmer, Waltham, MA). Data were expressed as percentage of control (untreated cells). Luciferase expression of untreated non-transfected cells were set as 100%. All experiments were performed in quadruplicate.

In vivo FVII assay

Six-to eight-week-old, female CD-1 mice (Charles River Laboratories) were administered ZL-siRNA formulations via tail vein injection at an siRNA concentration of 5 mg/kg (15:1 w:w ratio of ZL:siRNA) in a total volume of 200 µl. Control mice received an injection of 200 µL PBS. 48h after administration, animals were anesthetized with isoflurane. Blood was collected by submandibular cheek bleeding. Serum samples were obtained by allowing the blood to clot for 30 min and then centrifuging for 15 min at 15,000 rpm at 4 °C. The supernatant was collected and analyzed for serum levels of Factor VII protein using the Biophen VII colorimetric assay (Aniara, Mason, OH) according to manufacturer's instructions.[4] A standard curve was generated using serially diluted concentrations of PBS-treated animals. Serum levels of mice treated with siRNA-ZL formulations were expressed as percentage of PBS-control. Each group consisted of n=3 mice. DLinDMA served as a positive control.

ZL Liposomal Formulations Tested:

1. DOBAQ:Chol:DSPC:PEG-DMG (55:25:10:10)
2. DOBAQ:Chol:DSPC:PEG-DMG (40:40:10:10)
3. DOBAQ:CHEMS:PEG-DMG (60:30:10)
4. DOBAQ:CHEMS:PEG-DMG:Lactosylceramide (60:30:7:3)

Lactosylceramide was chosen as a hepatocyte targeting ligand that interacts with the asialoglycoprotein receptor. When incorporated in liposomes, lactosylceramide is known to promote liposomal accumulation in hepatocytes.[5]

In vivo cytokine induction by DOBAQ liposomes

Six-to eight-week-old, female CD-1 mice (Charles River Laboratories) were administered DOBAQ/Chol/DSPC/PEG-DMG (40:40:10:10) formulation via tail vein injection at an siRNA concentration of 5mg/kg (15:1 w:w ratio of ZL:siRNA) in a total volume of 200 µl. Control mice received an injection of 200 µL PBS. 48h after administration, animals

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were anesthetized with isoflurane. Blood was collected by submandibular cheek bleeding. Serum samples were obtained by allowing the blood to clot for 30 min and then centrifuging for 15 min at 15,000 RPM at 4C. The supernatant was collected and frozen at -80 °C. Serum samples were analyzed by Eve Technologies (Calgary, Alberta, Canada) using the Mouse 32-Plex Cytokine / Chemokine Panel.

siRNA Sequences

Lowercase = 2'-fluoro modified nucleotides
Asterisk = phosphorothioate linkage

Anti-Luciferase

Sense: 5' –GCUACAUUCUGGAGAGAUAdTdT-3'
Antisense: 5'-UAUGUCUCCAGAAUGUAGCdTdT-3'

Non-specific control provided by Pfizer

Factor VII

Sense: 5'-GGAucAucucAAGucuuAcT*T-3'
Antisense: 5'-GuAAGAcuuGAGAuGAuccT*T-3'

Non-specific: siGenome Non-Targeting siRNA #5 (Dharmacon, Lafeyette, CO)

Abbreviations

DOPC – 1,2-dioleoyl-*sn*-glycero-3-phosphocholine

DSPC - 1,2-distearyl-*sn*-glycero-3-phosphocholine

PEG-DMG – 1-(monomethoxypolyethyleneglycol)-2,3-dimyrystoylglycerol

Chol – Cholesterol

CHEMS – Cholesteryl Hemisuccinate

ANTS - 8-aminonophthalene-1,3,6-trisulfonate

DPX - p-xylene-bis-pyridinium bromide

Rho-PE - 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine-N-(lissamine rhodamine B sulfonyl) (ammonium salt)

NBD-PE - 1,2-dioleoyl-*sn*-glycero-3-phospho-L-serine-N-(7-nitro-2-1,3-benzoxadiazol-4-yl) (ammonium salt)

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Colloquial Naming Scheme for ZL

All ZL contain two ester-linked oleoyl chains (Di-Oleoyl). ZL were colloquially named as follows: DOPAQ and DOPAT contain a Propionic Acid head group and a Quaternary or Tertiary amine, respectively; DOMPAQ and DOMPAT contain a MethylPropionic Acid head group and a Quaternary or Tertiary amine, respectively; DOBAQ and DOBAT contain a Benzoic Acid head group and a Quaternary or Tertiary amine, respectively. The seventh lipid, DOAAQ, contains an Acetic Acid head group, and was synthesized with only a Quaternary amine.

Supplementary Tables and Figures

ZL	Ratio	Max Value (mV)	Min Value (mV)
DOMPAT	1:1	32.38 ± 2.11	-31.28 ± 4.42
	1:3	25.15 ± 1.52	-24.65 ± 0.95
	1:9	13.20 ± 0.98	-15.03 ± 1.30
DOPAT	1:1	38.90 ± 0.59	-43.85 ± 0.68
	1:3	30.00 ± 1.06	-29.18 ± 1.06
	1:9	13.40 ± 0.71	-16.00 ± 0.71
DOBAT	1:1	36.30 ± 2.30	-52.13 ± 1.25
	1:3	29.63 ± 1.34	-38.88 ± 1.60
	1:9	16.53 ± 0.46	-21.75 ± 0.64
DOMPAQ	1:1	32.75 ± 0.37	-10.80 ± 0.59
	1:3	25.25 ± 1.08	-6.47 ± 0.63
	1:9	12.48 ± 1.07	-6.26 ± 1.28
DOBAQ	1:1	35.48 ±1.91	-11.18 ± 1.10
	1:3	35.28 ± 0.64	-6.82 ± 0.68
	1:9	18.60 ± 1.13	-6.36 ± 0.66
DOAAQ	1:1	N.D.	N.D.
	1:3	1.13 ± 1.95	-6.78 ± 0.71
	1:9	N.D.	N.D.

Table S1: Maximum and minimum zeta potential data for ZL:DOPC liposomes at varying ratios. DOAAQ was not ionized in the given pH range, so only 1:3 data was collected

Formulation	Encapsulation efficiency (%)	Hydrodynamic diameter (nm)	Zeta potential (mV)
DOBAQ/DOPE/PEG-DMG (60/30/10)	83 ± 4%	75 ± 15 nm	- 3.1 ± 3 mV
DOPAT/DOPE/PEG-DMG (60/30/10)	91 ± 9%	91 ± 12 nm	- 5.7 ± 2 mV
DOAAQ/DOPE/PEG-DMG (60/30/10)	2 ± 2%	86 ± 8 nm	-4.2 ± 4 mV
DOPC/DOPE/PEG-DMG (60/30/10)	5 ± 3 %	79 ± 10 nm	-5.0 ± 1 mV

Table S2: siRNA encapsulation efficiency, size, and zeta potential data for ZL and control (DOPC) liposomes.

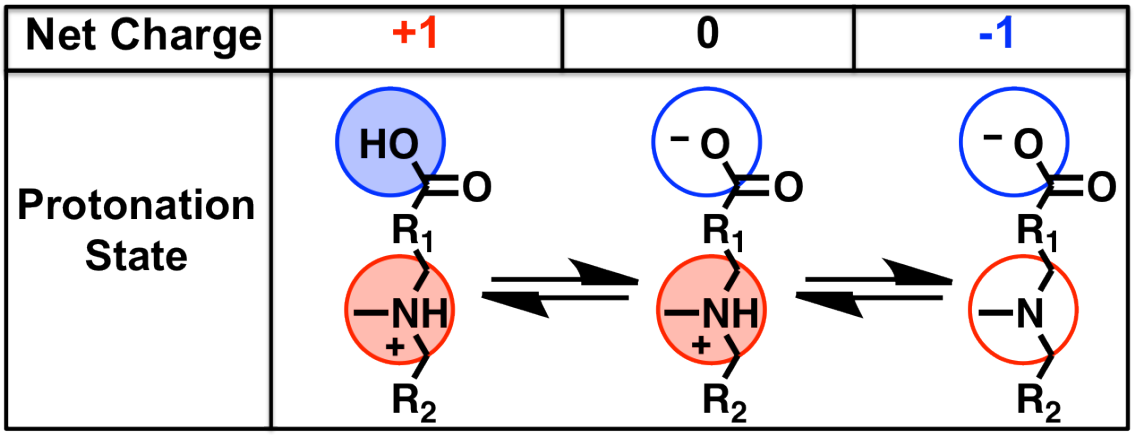

Figure S1: Theoretical ionization potential of tertiary ZL.

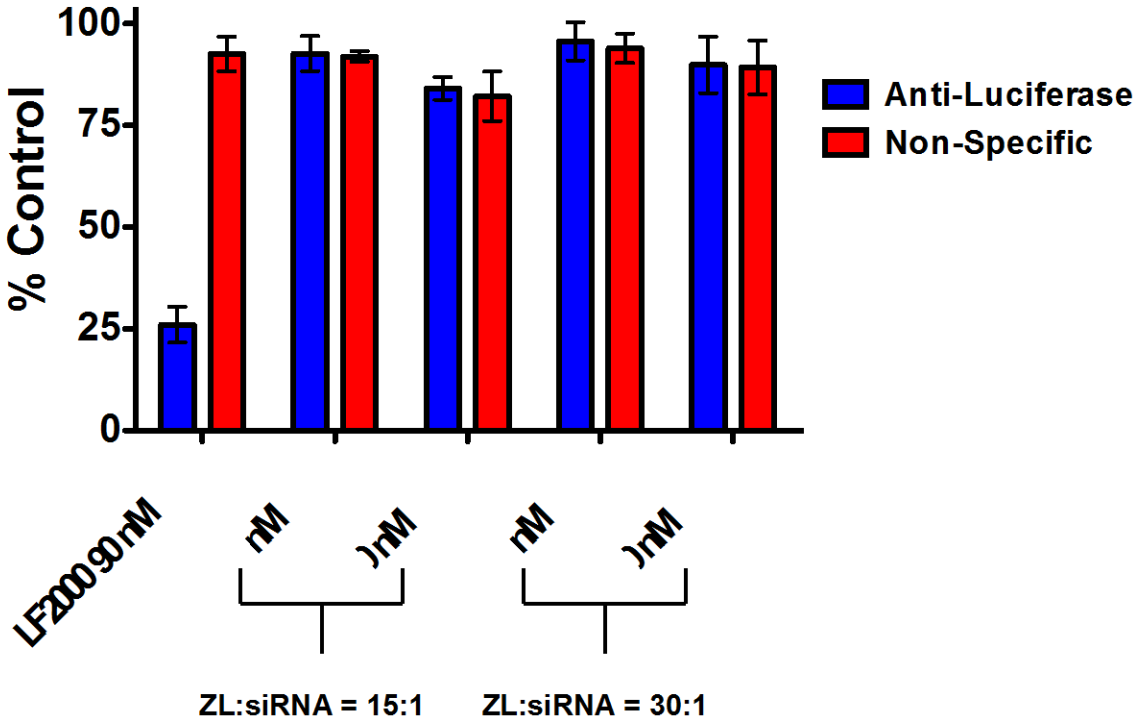

Figure S2: siRNA mediated knockdown of luciferase in Hela-Luc cells *in vitro.* Liposomal formulations are 65:34:1 DOBAQ:DOPE:PEG-DMG for all experiments. ZL:siRNA ratios are weight:weight ratios, and correspond to N:P ratios of 6.67 (15:1) and 13.34 (30:1).

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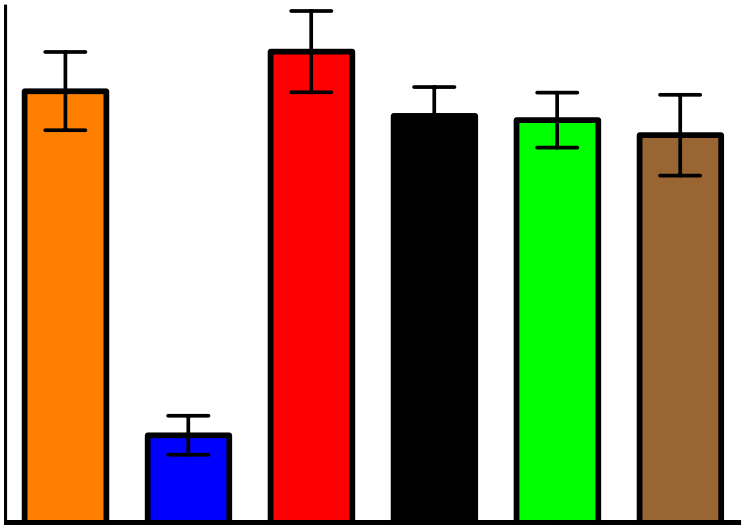

1. DOBAQ:Chol:DSPC:PEG-DMG (55:25:10:10)
2. DOBAQ:Chol:DSPC:PEG-DMG (40:40:10:10)
3. DOBAQ:CHEMS:PEG-DMG (60:30:10)
4. DOBAQ:CHEMS:PEG-DMG:Lactosylceramide (60:30:7:3)

Figure S3: siRNA mediated knockdown of Factor VII in CD-1 mice using ZL liposomes. All doses were 5 mg/kg total siRNA.

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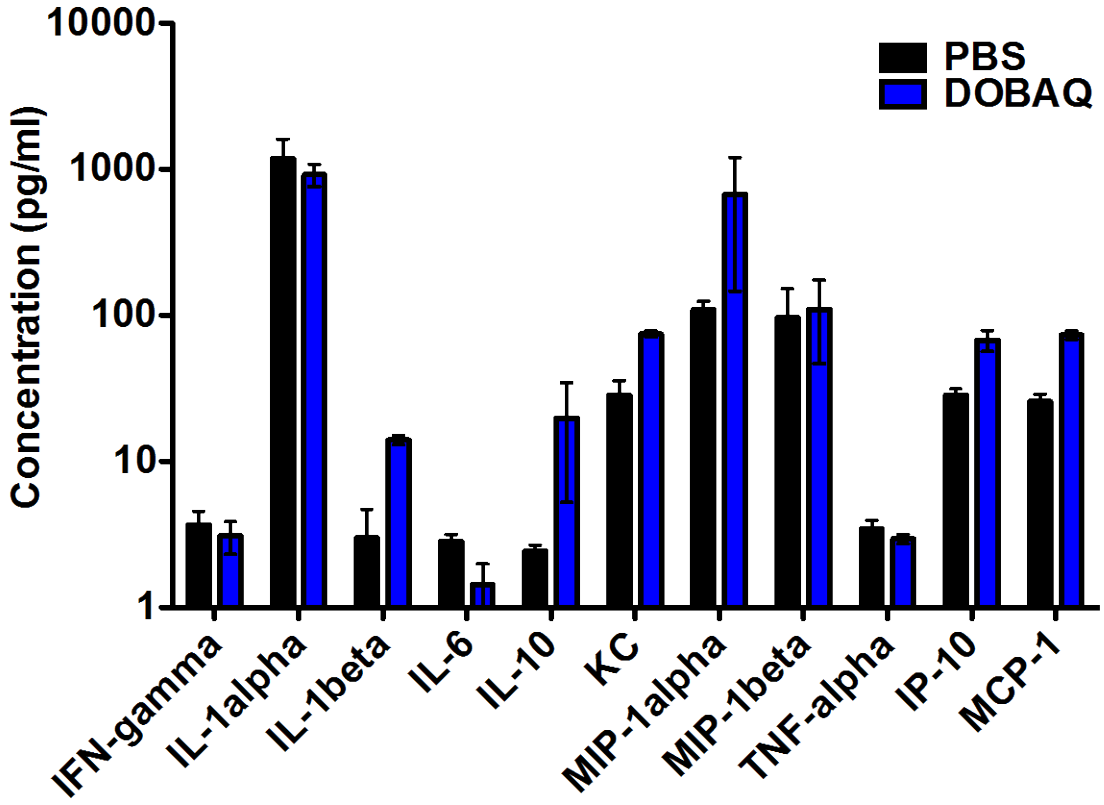

Figure S4: Cytokine induction by DOBAQ:Chol:DSPC:PEG-DMG (40:40:10:10) liposomes in CD-1 mice (5 mg/kg siRNA, 15:1 w:w ZL:siRNA). DOBAQ containing liposomes show no increase in cytokine induction compared to PBS 6 hours after injection.

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