Supporting Information

Molecular tuning of the vitamin E-scaffold pH-sensitive and reductive cleavable lipid-like material for accelerated *in vivo* hepatic siRNA delivery

AUTHORS

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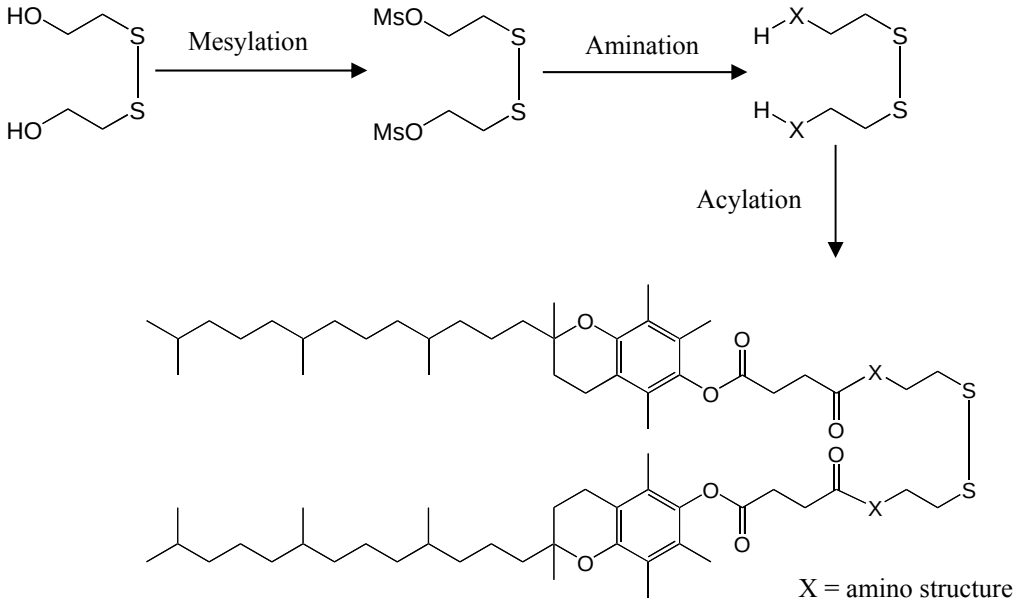
S1 Syntheses of ssPalms

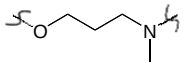
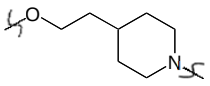
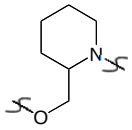
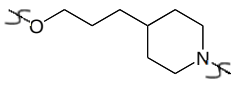
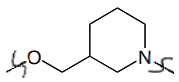
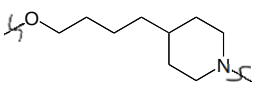
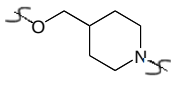
S1.1. General Procedures

All reagents were obtained from commercial sources and were used without further purification. Thin layer chromatography was performed on Merck TLC plates silica gel 60. ^{1}H-NMR spectra were recorded on a JEOL ECA600 (^{1}H 600MHz) spectrometer.

S1.2. Syntheses of ssPalms

ssPalms were synthesized according to the method outlined in Scheme S1.2.



lipid name	X (amino structure)	lipid name	X (amino structure)
ssPalmE		ssPalmE-P4-C2	
ssPalmE-P2		ssPlamE-P4-C3	
ssPalmE-P3		ssPalmE-P4-C4	
ssPalmE-P4-C1			

Scheme S1.2 Syntheses of ssPalm series

S1.2.1. Synthesis of dimesylate compound (Mesylation)

2,2'-Dithiodiethanol (15.0 g, 97 mmol) was dissolved in acetonitrile (143 mL) at 20-25^{o}C. Triethylamine (33.8 g, 328 mmol) was added to the solution, which was then stirred at 0-10ºC for 5 minutes. The mixture was treated with methanesulfonyl chloride (34.5 g, 300 mmol) at a rate such that temperature did not exceed 20^{o}C. The reaction was stirred for 3 hours, and the complete consumption of 2,2'-dithiodiethanol was confirmed by TLC analysis (eluent: chloroform/methanol = 85/15 (v/v)). After the quenching of the reaction by the addition of ethanol (29 mL), insoluble materials were removed by filtration. To the filtrate were added dichloromethane (150 mL) and a 10% aqueous sodium hydrogen carbonate solution (150 mL), and the mixture was stirred for 5 min. After standing for 10 minutes, the aqueous layer was removed. The organic layer was washed with water (4×150 mL) and then dried by adding sodium sulfate (4.5 g), filtered and evaporated to give the dimesylate compound (29.4 g, 97%) as a brown soild.

S1.2.2. Synthesis of ssPalmE

(Amination)

The dimesylate compound (5.0 g, 16 mmol) was dissolved in acetonitrile (127 mL) at 20-25ºC, and then potassium carbonate (5.5 g, 40 mmol) was added. The mixture was stirred at 20-25ºC for 5 minutes. 3-Methylamino-1-propanol (14.4 g, 160 mmol) was dissolved in acetonitrile (18 mL) at 25ºC, and added to the dimesylate compound/acetonitrile solution. The reaction was stirred at 25-35 ºC for 9 hours, and complete consumption of mesylate compound was confirmed by TLC analysis (eluent : chloroform/methanol/28% aqueous ammonia = 80/20/2 (v/v/v)). Potassium carbonate was removed by filtration, and the filtrate was evaporated to give a brown liquid. The obtained brown liquid was dissolved in chloroform (132 mL), and washed with 10% brine (5×132 mL). The organic layer was dried by adding magnesium sulfate (2.5 g), filtered and evaporated to yield diamino

compound (4.3 g, 91%) as a pale brown liquid.

(Acylation)

The diamino compound (3.0 g, 10 mmol) and D-α-tocopherol succinate (11.8 g, 22 mmol) were dissolved in chloroform (30 mL). 4-Dimethylamino pyridine (0.49 g, 4.0 mmol) and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (5.4 g, 28 mmol) were added to the solution. The reaction was stirred at 25-35ºC for 12 hours, and the complete consumption of D-α-tocopherol succinate was confirmed by TLC analysis (eluent : chloroform/methanol = 90/10 (v/v)). The reaction mixture was evaporated to give a pale brown liquid. The concentrate was dissolved in hexane (77 mL), and acetonitrile (39 mL) was added. The hexane layer was recovered, and evaporated to give crude ssPalmE as a pale brown liquid. The crude materials was purified by silica gel column chromatography (elute : chloroform/methanol = 99/1 (v/v)-97/3 (v/v)) to yield ssPalmE (6.6 g, 50%) as a pale yellow liquid. ^{1}H NMR (600 MHz, $CDCl_3$): δ (ppm) = 0.85-0.90 (m, 24H, C*H*$_3$-CH$_2$-, C*H*$_3$-CH-), 1.00-1.83 (m, 56H, C*H*$_3$-C-, (CH$_3$)$_2$C*H*-(C*H*$_2$)$_3$-C*H*(CH$_3$)-(C*H*$_2$)$_3$-C*H*(CH$_3$)-(C*H*$_2$)$_3$-, -O-(CH$_3$)C-C*H*$_2$-CH$_2$-, C(O)-O-CH$_2$-C*H*$_2$-), 1.95-2.15 (s, 18H, Ar-C*H*$_3$), 2.20-2.30 (s, 6H, C*H*$_3$-N-), 2.40-2.50 (t, 4H, -CH$_2$-CH$_2$-C*H*$_2$-N-), 2.55-2.63 (t, 4H, -CH$_2$-C*H*$_2$-C=C(CH$_3$)-), 2.65-2.69 (m, 4H, -N-CH$_2$-C*H*$_2$-S-), 2.70-2.81 (m, 8H, -N-C*H*$_2$-CH$_2$-S-, Ar-O-C(O)-C*H*$_2$-CH$_2$-), 2.90-2.95 (t, 4H, Ar-O-C(O)-CH$_2$-C*H*$_2$-), 4.10-4.25 (t, 4H, -O-C*H*$_2$-CH$_2$-CH$_2$-N-).

S1.2.3. Synthesis of ssPalmE-P2

(Amination)

The dimesylate compound (1.2 g, 3.9 mmol) was dissolved in acetonitrile (46 mL) at 20-25ºC, and potassium carbonate (1.3 g, 9.7 mmol) was then added. The mixture was stirred at 20-25ºC for 5 minutes. 2-Piperidinemethanol (4.5 g, 39 mmol) was dissolved in acetonitrile (4.6 mL) at 25ºC, and added to the dimesylate compound/acetonitrile solution. The reaction was stirred at 25-35ºC for 13

hours, and the complete consumption of the mesylate compound was confirmed by TLC analysis (eluent : chloroform/methanol/28% aqueous ammonia = 80/20/2 (v/v/v)). Potassium carbonate was removed by filtration, and the filtrate was evaporated to give a brown liquid. The resulting brown liquid was dissolved in chloroform (39 mL), and washed with 10% brine (5×39 mL). The organic layer was dried by adding magnesium sulfate (0.60 g), filtered and evaporated to yield diamino compound (1.2 g, 89%) as a brown liquid.

(Acylation)

The diamino compound (0.80 g, 2.3 mmol) and D-α-tocopherol succinate (2.6 g, 4.8 mmol) were dissolved in chloroform (8.0 mL). 4-Dimethylamino pyridine (0.11 g, 0.92 mmol) and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (1.3 g, 6.9 mmol) were added to the solution. The reaction was stirred at 25-35ºC for 4 hours, and the complete consumption of D-α-tocopherol succinate was confirmed by TLC analysis (eluent : chloroform/methanol = 90/10 (v/v)). The reaction mixture was evaporated to give brown liquid. The concentrate was dissolved in hexane (60 mL), and acetonitrile (30 mL) was added. The hexane layer was recovered, and evaporated to give crude ssPalmE-P2 as a pale brown liquid. The crude materials was purified by silica gel column chromatography (elute : chloroform/methanol = 99/1 (v/v)) to yield ssPalmE-P2 (0.58 g, 20%) as a pale yellow liquid. ^{1}H NMR (600 MHz, $CDCl_3$): δ (ppm) = 0.83-0.88 (m, 24H, C*H*$_3$-CH$_2$-, C*H*$_3$-CH-), 1.02-1.80 (m, 64H, -CH-N-CH$_2$-C*H*$_2$-C*H*$_2$-C*H*$_2$-, C*H*$_3$-C-, (CH$_3$)$_2$C*H*-(C*H*$_2$)$_3$-C*H*(CH$_3$)-(C*H*$_2$)$_3$-C*H*(CH$_3$)-(C*H*$_2$)$_3$-, -O-(CH$_3$)C-C*H*$_2$-CH$_2$-), 1.95-2.10 (s, 18H, Ar-C*H*$_3$), 2.30-2.36 (m, 2H, -C(O)-O-CH$_2$-C*H*-), 2.55-2.60 (m, 6H, -CH$_2$-C*H*$_2$-C=C(CH$_3$)-, -N-C*H*$^{(a)}$$_2$-CH$_2$-), 2.76-2.94 (m, 16H, -N-C*H*$_2$-C*H*$_2$-S-, Ar-O-C(O)-C*H*$_2$-C*H*$_2$-), 2.96-3.07 (m, 2H, -N-C*H*$^{(e)}$$_2$-CH$_2$-), 4.12-4.27 (m, 4H, -C(O)-O-C*H*$_2$-CH-CH$_2$-).

S1.2.4. Synthesis of ssPalmE-P3

(Amination)

The dimesylate compound (1.2 g, 3.9 mmol) was dissolved in acetonitrile (31 mL) at 20-25ºC, and potassium carbonate (1.3 g, 9.7 mmol) was then added. The mixture was stirred at 20-25ºC for 5 minutes. 3-Piperidinemethanol (4.5 g, 39 mmol) was dissolved in acetonitrile (4.6 mL) at 25ºC, and added to the dimesylate compound/acetonitrile solution. The reaction was stirred at 25-35 ºC for 6 hours, and the complete consumption of the mesylate compound was confirmed by TLC analysis (eluent : chloroform/methanol/28% aqueous ammonia = 80/20/2 (v/v/v)). Potassium carbonate was removed by filtration, and the filtrate was evaporated to give a brown liquid. The obtained brown liquid was dissolved in chloroform (41 mL), and washed with 10% brine (5×41 mL). The organic layer was dried over magnesium sulfate (0.60 g), filtered and evaporated to yield the diamino compound (1.3 g, 96%) as a brown liquid.

(Acylation)

The diamino compound (0.60 g, 1.7 mmol) and D-α-tocopherol succinate (1.9 g, 3.6 mmol) were dissolved in chloroform (6.0 mL). 4-Dimethylamino pyridine (0.084 g, 0.69 mmol) and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (0.99 g, 5.2 mmol) were added to the solution. The reaction was stirred at 25-35ºC for 7 hours, and the complete consumption of D-α-tocopherol succinate was confirmed by TLC analysis (eluent : chloroform/methanol = 90/10 (v/v)). The reaction mixture was evaporated to give a brown liquid. The concentrate was dissolved in hexane (44 mL), and acetonitrile (22 mL) was added thereto for extraction washing. The hexane layer was recovered, and evaporated to give crude ssPalmE-P3 as a pale brown liquid. The crude materials was purified by silica gel column chromatography (elute : chloroform/methanol = 99/1 (v/v)-98/2 (v/v)) to yield ssPalmE-P3 (0.69 g, 30%) as a pale yellow liquid. ^{1}H NMR (600 MHz, $CDCl_3$): δ (ppm) = 0.83-0.88 (m, 24H, C*H*$_3$-CH$_2$-, C*H*$_3$-CH-), 1.02-1.80 (m, 62H, -C*H*-CH$_2$-N-CH$_2$-C*H*$_2$-C*H*$_2$-, C*H*$_3$-C-, (CH$_3$)$_2$C*H*-(C*H*$_2$)$_3$-C*H*(CH$_3$)-(C*H*$_2$)$_3$-C*H*(CH$_3$)-(C*H*$_2$)$_3$-, -O-(CH$_3$)C-C*H*$_2$-CH$_2$-), 1.95-2.10 (m, 22H, Ar-C*H*$_3$, -CH-C*H*$^{(a)}$$_2$-N-C*H*$^{(a)}$$_2$-CH$_2$-CH$_2$-), 2.55-2.60 (t,

4H, -CH_2-CH_2-C=C(CH_3)-), 2.63-2.66 (m, 4H, -N-CH_2-CH_2-S-), 2.73-2.85 (m, 12H, -N-CH_2-CH_2-S-, Ar-O-C(O)-CH_2-CH_2-, -CH-C$H^{(e)}_2$-N-C$H^{(e)}_2$-CH_2-CH_2-), 2.90-2.95 (t, 4H, Ar-O-C(O)-CH_2-CH_2-), 3.94-4.04 (m, 4H, -C(O)-O-CH_2-CH-CH_2-).

S1.2.5. Synthesis of ssPalmE-P4-C1

(Amination)

The dimesylate compound (1.2 g, 3.9 mmol) was dissolved in acetonitrile (31 mL) at 20-25ºC, and potassium carbonate (1.3 g, 9.7 mmol) was then added. The mixture was stirred at 20-25ºC for 5 minutes. 4-Piperidinemethanol (4.5 g, 39 mmol) was dissolved in acetonitrile (4.6 mL) at 25ºC, and added to the dimesylate compound/acetonitrile solution. The reaction was stirred at 25-35ºC for 4 hours, and the complete consumption of the mesylate compound was confirmed by TLC analysis (eluent : chloroform/methanol/28% aqueous ammonia = 80/20/2 (v/v/v)). Potassium carbonate was removed by filtration, and the filtrate was evaporated to give a brown liquid. The resulting brown liquid was dissolved in chloroform (42 mL), and washed with 10% brine (4×42 mL). The organic layer was dried over magnesium sulfate (0.6 g), filtered and evaporated to yield the diamino compound (1.2 g, 89%) as a brown liquid.

(Acylation)

The diamino compound (0.60 g, 1.7 mmol) and D-α-tocopherol succinate (1.9 g, 3.6 mmol) were dissolved in chloroform (6.0 mL). 4-Dimethylamino pyridine (0.084 g, 0.69 mmol) and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (0.99 g, 5.2 mmol) were added to the solution. The reaction was stirred at 25-35ºC for 7 hours, and the complete consumption of D-α-tocopherol succinate was confirmed by TLC analysis (eluent : chloroform/methanol = 90/10 (v/v)). The reaction mixture was evaporated to give a brown liquid. The concentrate was dissolved in hexane (42 mL), and acetonitrile (21 mL) was added thereto for extraction washing. The hexane layer was recovered, and evaporated to give crude ssPalmE-P4-C1 as a pale brown liquid. The crude

materials was purified by silica gel column chromatography (elute : chloroform/methanol = 99/1 (v/v)-98/2 (v/v)) to yield ssPalmE-P4-C1 (1.3 g, 55%) as a pale yellow liquid. ^{1}H NMR (600 MHz, $CDCl_3$): δ (ppm) = 0.83-0.88 (m, 24H, C*H*$_3$-CH$_2$-, C*H*$_3$-CH-), 1.02-1.74 (m, 62H, -C*H*-C*H*$_2$-CH$_2$-N-, C*H*$_3$-C-, (CH$_3$)$_2$C*H*-(C*H*$_2$)$_3$-C*H*(CH$_3$)-(C*H*$_2$)$_3$-C*H*(CH$_3$)-(C*H*$_2$)$_3$-, -O-(CH$_3$)C-C*H*$_2$-CH$_2$-), 1.95-2.10 (m, 22H, Ar-C*H*$_3$, -CH-CH$_2$-CH$_2$-C*H*$^{(a)}$$_2$-N-), 2.55-2.60 (t, 4H, -CH$_2$-C*H*$_2$-C=C(CH$_3$)-), 2.63-2.67 (m, 4H, -N-CH$_2$-C*H*$_2$-S-), 2.74-2.77 (t, 4H, Ar-O-C(O)-C*H*$_2$-CH$_2$-), 2.81-2.84 (m, 4H, -N-C*H*$_2$-CH$_2$-S-), 2.90-2.95 (m, 8H, -CH-CH$_2$-CH$_2$-C*H*$^{(e)}$$_2$-N-, Ar-O-C(O)-CH$_2$-C*H*$_2$-), 3.95-3.98 (d, 4H, -C(O)-O-C*H*$_2$-CH$_2$-CH-).

S1.2.6. Synthesis of ssPalmE-P4-C2

(Amination)

The dimesylate compound (3.0 g, 9.7 mmol) was dissolved in acetonitrile (69 mL) at 20-25ºC, and potassium carbonate (2.0 g, 15 mmol) was then added. The mixture was stirred at 20-25ºC for 5 minutes. 4-Piperidineethanol (3.8 g, 29 mmol) was dissolved in acetonitrile (7.7 mL) at 25ºC, and added to the dimesylate compound/acetonitrile solution. The reaction was stirred at 25-35ºC for 19 hours, and the complete consumption of the mesylate compound was confirmed by TLC analysis (eluent : chloroform/methanol/28% aqueous ammonia = 80/20/2 (v/v/v)). Potassium carbonate was removed by filtration, and the filtrate was evaporated to give a brown liquid. The resulting brown liquid was dissolved in chloroform (51 mL), and washed with 5% sodium hydrogen carbonate aqueous (2×51 mL). The organic layer was dried over magnesium sulfate (1.5 g), filtered and evaporated to yield the diamino compound (3.6 g, 99%) as a brown liquid.

(Acylation)

The diamino compound (0.90 g, 2.4 mmol) and D-α-tocopherol succinate (2.5 g, 4.8 mmol) were dissolved in chloroform (9.0 mL). 4-Dimethylamino pyridine (0.12 g, 0.96 mmol) and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (1.4 g, 7.2 mmol) were added to the

solution. The reaction was stirred at 25-35ºC for 8 hours, and the complete consumption of D-α-tocopherol succinate was confirmed by TLC analysis (eluent : chloroform/methanol = 90/10 (v/v)). The reaction mixture was evaporated to give a brown liquid. The concentrate was dissolved in hexane (56 mL), and acetonitrile (28 mL) was added thereto for extraction washing. The hexane layer was recovered, and evaporated to give the crude ssPalmE-P4-C2 as a pale brown liquid. The crude materials was purified by silica gel column chromatography (elute : chloroform/methanol = 99/1 (v/v)-98/2 (v/v)) to yield ssPalmE-P4-C2 (1.3 g, 39%) as a pale yellow liquid. ^{1}H NMR (600 MHz, $CDCl_3$): δ (ppm) = 0.83-0.88 (m, 24H, C*H*$_3$-CH$_2$-, C*H*$_3$-CH-), 1.05-1.81 (m, 66H, -C*H*-C*H*$_2$-CH$_2$-N-, C*H*$_3$-C-, (CH$_3$)$_2$C*H*-(C*H*$_2$)$_3$-C*H*(CH$_3$)-(C*H*$_2$)$_3$-C*H*(CH$_3$)-(C*H*$_2$)$_3$-, -C(O)-O-CH$_2$-C*H*$_2$-CH-, -O-(CH$_3$)C-C*H*$_2$-), 1.95-2.10 (m, 22H, Ar-C*H*$_3$, -CH-CH$_2$-CH$_2$-C*H*$^{(a)}$$_2$-N-), 2.55-2.60 (t, 4H, -CH$_2$-C*H*$_2$-C=C(CH$_3$)-), 2.62-2.66 (m, 4H, -N-CH$_2$-C*H*$_2$-S-), 2.73-2.77 (t, 4H, Ar-O-C(O)-C*H*$_2$-CH$_2$-), 2.80-2.84 (m, 4H, -N-C*H*$_2$-CH$_2$-S-), 2.86-2.95 (m, 8H, -CH-CH$_2$-CH$_2$-C*H*$^{(e)}$$_2$-N-, Ar-O-C(O)-CH$_2$-C*H*$_2$-), 4.12-4.17 (t, 4H, -C(O)-O-C*H*$_2$-CH$_2$-CH-).

S1.2.7. Synthesis of ssPalmE-P4-C3

(Amination)

The dimesylate compound (0.80 g, 2.6 mmol) was dissolved in acetonitrile (18 mL) at 20-25ºC, and potassium carbonate (0.89 g, 6.4 mmol) was then added. The mixture was stirred at 20-25ºC for 5 minutes. 4-Piperidinepropanol (1.1 g, 7.7 mmol) was dissolved in acetonitrile (2.0 mL) at 25ºC, and added to the dimesylate compound/acetonitrile solution. The reaction was stirred at 25-35ºC for 21 hours, and the complete consumption of the mesylate compound was confirmed by TLC analysis (eluent : chloroform/methanol/28% aqueous ammonia = 80/20/2 (v/v/v)). Potassium carbonate was removed by filtration, and the filtrate was evaporated to give a brown liquid. The obtained brown liquid was dissolved in chloroform (14 mL), and washed with 5% sodium hydrogen carbonate aqueous (2×14 mL). The organic layer was dried over magnesium sulfate (0.40 g), filtered and

evaporated to yield diamino compound (0.70 g, 67%) as a brown liquid.

(Acylation)

The diamino compound (0.60 g, 1.5 mmol) and D-α-tocopherol succinate (1.6 g, 3.0 mmol) were dissolved in chloroform (6.0 mL). 4-Dimethylamino pyridine (0.072 g, 0.59 mmol) and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (0.85 g, 4.4 mmol) were added to the solution. The reaction was stirred at 25-35ºC for 6 hours, and the complete consumption of D-α-tocopherol succinate was confirmed by TLC analysis (eluent : chloroform/methanol = 90/10 (v/v)). The reaction mixture was evaporated to give brown liquid. The concentrate was dissolved in hexane (38 mL), and acetonitrile (19 mL) was added. The hexane layer was recovered, and evaporated to give the crude ssPalmE-P4-C3 as a pale brown liquid. The crude materials was purified by silica gel column chromatography (elute : chloroform/methanol = 99/1 (v/v)-98/2 (v/v)) to yield ssPalmE-P4-C3 (1.0 g, 48%) as a pale yellow liquid. ^{1}H NMR (600 MHz, $CDCl_3$): δ (ppm) = 0.83-0.88 (m, 24H, C*H*$_3$-CH$_2$-, C*H*$_3$-CH-), 1.05-1.83 (m, 70H, -C*H*-C*H*$_2$-CH$_2$-N-, C*H*$_3$-C-, $(CH_3)_2$C*H*-(C*H*$_2$)$_3$-C*H*(CH$_3$)-(C*H*$_2$)$_3$-C*H*(CH$_3$)-(C*H*$_2$)$_3$-, -C(O)-O-CH$_2$-C*H*$_2$-C*H*$_2$-CH-, -O-(CH$_3$)C-C*H*$_2$-), 1.95-2.10 (m, 22H, Ar-C*H*$_3$, -CH-CH$_2$-CH$_2$-C*H*$^{(a)}$$_2$-N-), 2.55-2.60 (t, 4H, -CH$_2$-C*H*$_2$-C=C(CH$_3$)-), 2.62-2.66 (m, 4H, -N-CH$_2$-C*H*$_2$-S-), 2.73-2.77 (t, 4H, Ar-O-C(O)-C*H*$_2$-CH$_2$-), 2.80-2.84 (m, 4H, -N-C*H*$_2$-CH$_2$-S-), 2.86-2.95 (m, 8H, -CH-CH$_2$-CH$_2$-C*H*$^{(e)}$$_2$-N-, Ar-O-C(O)-CH$_2$-C*H*$_2$-), 4.07-4.11 (t, 4H, -C(O)-O-C*H*$_2$-CH$_2$-CH-).

S1.2.8. Synthesis of ssPalmE-P4-C4

(Amination)

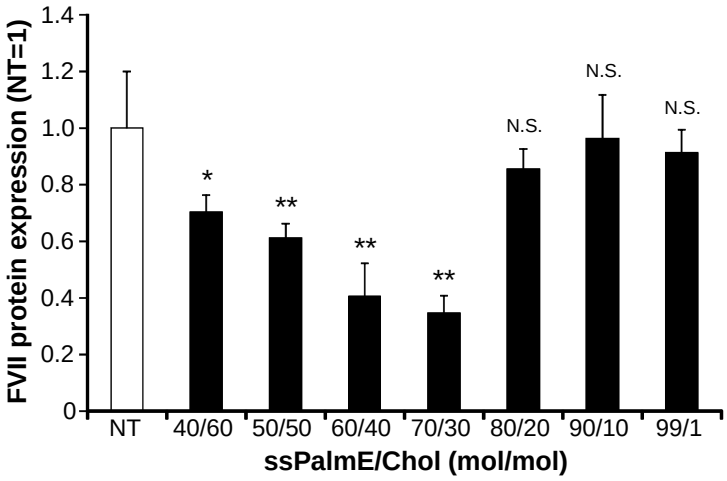
The dimesylate compound (0.80 g, 2.6 mmol) was dissolved in acetonitrile (18 mL) at 20-25ºC, and then potassium carbonate (0.89 g, 6.4 mmol) was added. The mixture was stirred at 20-25ºC for 5 minutes. 4-Piperidinebutanol (1.2 g, 7.7 mmol) was dissolved in acetonitrile (2.0 mL) at 25ºC, and added to the dimesylate compound/acetonitrile solution. The reaction was stirred at 25-35ºC for 19

hours, and the complete consumption of the mesylate compound was confirmed by TLC analysis (eluent : chloroform/methanol/28% aqueous ammonia = 80/20/2 (v/v/v)). Potassium carbonate was removed by filtration, and the filtrate was evaporated to give a brown liquid. The resulting brown liquid was dissolved in chloroform (14 mL), and washed with 5% sodium hydrogen carbonate aqueous (2×14 mL). After drying the organic layer over magnesium sulfate (0.40 g), it was filtered and the filtrate evaporated to yield the diamino compound (1.1 g, 99%) as a brown liquid.

(Acylation)

The diamino compound (0.60 g, 1.4 mmol) and D-α-tocopherol succinate (1.5 g, 2.8 mmol) were dissolved in chloroform (6.0 mL). 4-Dimethylamino pyridine (0.068 g, 0.56 mmol) and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (0.80 g, 4.2 mmol) were added to the solution. The reaction was stirred at 25-35ºC for 6 hours, and the complete consumption of D-α-tocopherol succinate was confirmed by TLC analysis (eluent : chloroform/methanol = 90/10 (v/v)). The reaction mixture was evaporated to give brown liquid. The concentrate was dissolved in hexane (36 mL), and acetonitrile (18 mL) was added. The hexane layer was recovered, and evaporated to give crude ssPalmE-P4-C4 as a pale brown liquid. The crude materials was purified by silica gel column chromatography (elute : chloroform/methanol = 99/1 (v/v)-98/2 (v/v)) to yield ssPalmE-P4-C4 (0.80 g, 40%) as a pale yellow liquid. ^{1}H NMR (600 MHz, $CDCl_3$): δ (ppm) = 0.83-0.88 (m, 24H, C*H*$_3$-CH$_2$-, C*H*$_3$-CH-), 1.05-1.80 (m, 74H, -C*H*-C*H*$_2$-CH$_2$-N-, C*H*$_3$-C-, (CH$_3$)$_2$C*H*-(C*H*$_2$)$_3$-C*H*(CH$_3$)-(C*H*$_2$)$_3$-C*H*(CH$_3$)-(C*H*$_2$)$_3$-, -C(O)-O-CH$_2$-C*H*$_2$-C*H*$_2$-C*H*$_2$-CH-, -O-(CH$_3$)C-C*H*$_2$-), 1.95-2.10 (m, 22H, Ar-C*H*$_3$, -CH-CH$_2$-CH$_2$-C*H*$^{(a)}$$_2$-N-), 2.55-2.60 (t, 4H, -CH$_2$-C*H*$_2$-C=C(CH$_3$)-), 2.62-2.66 (m, 4H, -N-CH$_2$-C*H*$_2$-S-), 2.73-2.77 (t, 4H, Ar-O-C(O)-C*H*$_2$-CH$_2$-), 2.80-2.84 (m, 4H, -N-C*H*$_2$-CH$_2$-S-), 2.86-2.95 (m, 8H, -CH-CH$_2$-CH$_2$-C*H*$^{(e)}$$_2$-N-, Ar-O-C(O)-CH$_2$-C*H*$_2$-), 4.06-4.11 (t, 4H, -C(O)-O-C*H*$_2$-CH$_2$-CH-).

Supplemental Figure S1



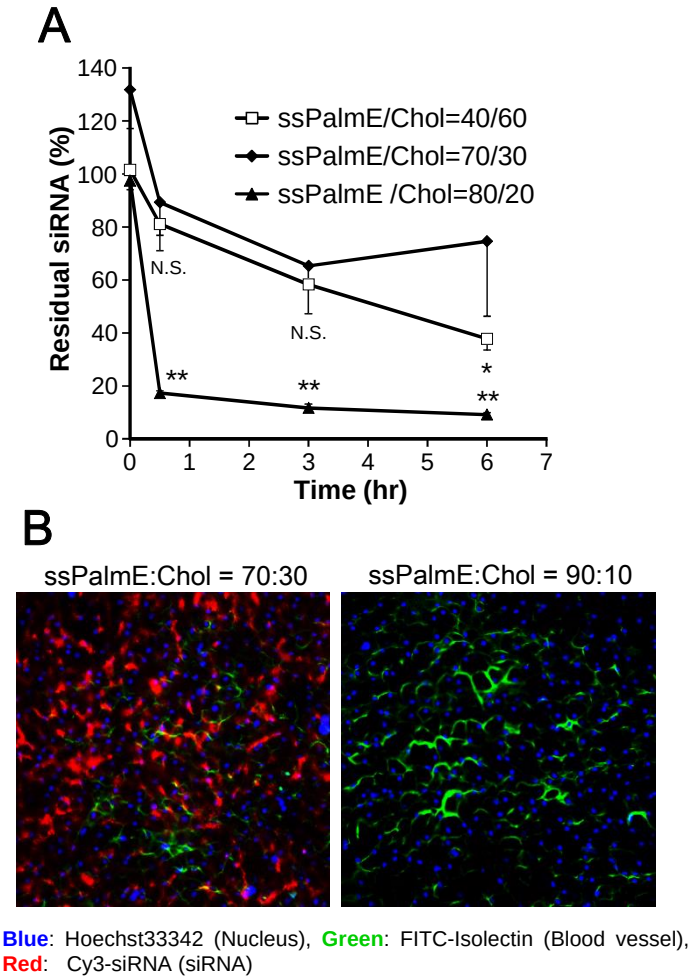
Supplemental Figure S1

Optimization of ssPalmE to Chol ratio to maximize a gene knockdown efficacy against FVII.

Influence of ssPalmE content on FVII gene silencing was monitored. A series of $LNP_{ssPalmE}$ containing 40 to 99 mol% of ssPalmE were prepared, and intravenously administered. The total lipid amount was adjusted by the Chol. Additionally, 3 mol% of DMG-PEG_{2000} was added to stabilize the LNPs. At 24 h after the administration at a dose of 1.5 mg siRNA (2'-OH) /kg, the serum FVII was monitored.

The gene knockdown activity increased up to 70 mol% of ssPalmE, but was drastically impaired when ssPalmE were increased to >80 mol%. Collectively, the $LNP_{ssPalmE}$ composed of ssPalmE:Chol = 70:30 is the optimum lipid composition.

Supplemental Figure S2



Supplemental Figure S2

Stability of siRNA encapsulated in LNPs in serum.

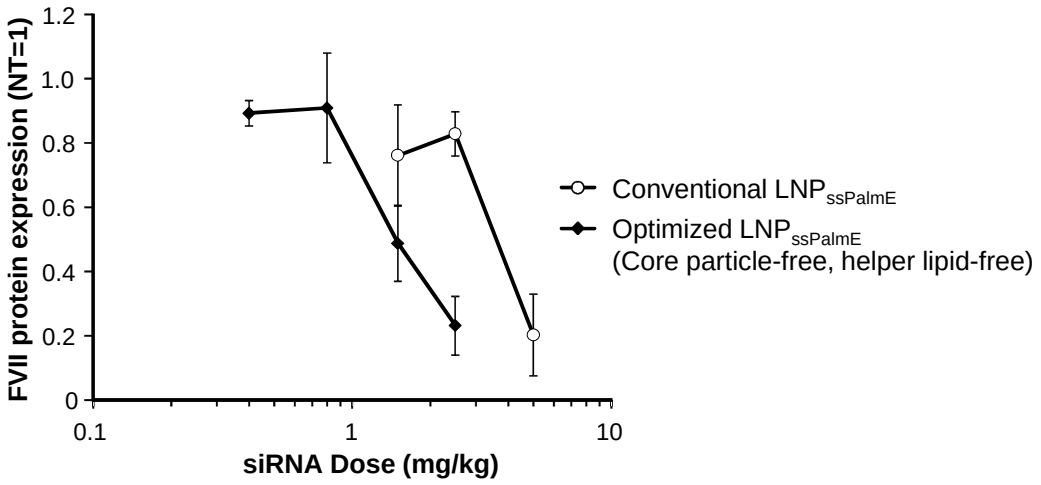
A. A 50 μL of LNPs (containing 400 nM of siRNA (2'-OH)), that were prepared with indicated lipid composition were incubated with 200 μL of FBS (finally 80%) at 37°C. At indicated time, the siRNA samples were rapidly frozen in liquid N_2. After the purification of siRNA, the intact siRNA was quantified by stem-loop PCR. Reverse transcription reactions were performed with TaqMan MicroRNA Reverse Transcription kit (Applied Biosystems, Carlsbad, CA). Subsequently, the number of siRNA copies was quantified by using TaqMan PCR Master Mix (Applied Biosystems) by means of Agilent Stratagene Mx3000P QPCR System (Agilent, Foster City, CA, USA). The resistance against 80% FBS were drastically decreased when the mol% of ssPalmE exceeds >80%.

B. Accumulation of siRNA in the liver. $LNP_{ssPalmE}$ encapsulating Cy3-labeled siRNA were administered into male ICR mice. At 30 min after intravenously injection of LNPs, a piece of liver section was collected as described in the Materials and Methods. The liver section was stained with 20 μg/mL of Hoechst33342 and 0.2 mg/mL of Griffonia simplicifolia lectin isolectin B4 (FITC–GS-IB4: Vector Laboratories, Burlingame, CA, USA) for 1 h at 4°C. The tissue was observed by a Nikon A1 microscope equipped with a water immersion objective lens (Plan Apo 60× 1.20 PFS WI). The signals for Hoechst33342, FITC–GS-IB4 and Cy3-labeled siRNA were pseudo-colored in blue, green and red, respectively.

The hepatic accumulation of $LNP_{ssPalmE}$ was drastically impaired in $LNP_{ssPalmE}$ composed of ssPalmE:Chol = 90:10, while that prepared by ssPalmE:Chol = 70:30 were efficiently accumulated in hepatocyte.

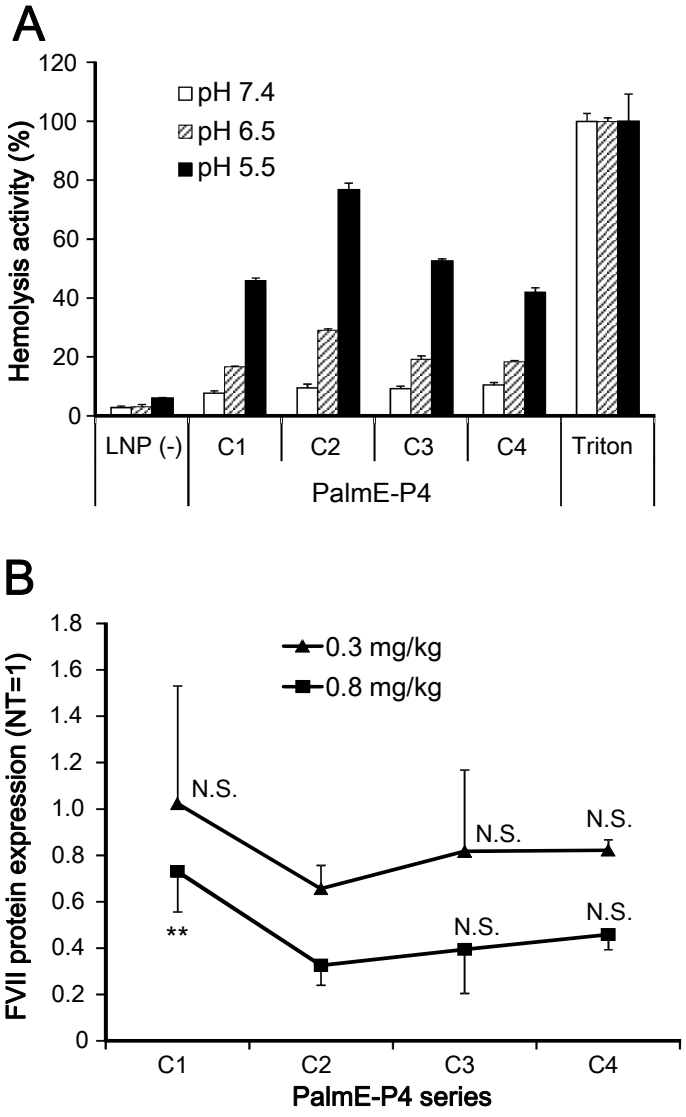
Collectively, at least 30 mol% of cholesterol is required to stabilize $LNP_{ssPalmE}$ in serum.

Supplemental Figure S3



Supplemental Figure S3
Comparison of the gene knockdown efficiencies between conventional $LNP_{ssPalmE}$ (core particle(+)/helper lipid(+)) and Optimized $LNP_{ssPalmE}$ (core particle(-)/helper lipid(-))
in vivo gene knockdown efficacies of conventional $LNP_{ssPalmE}$ (**Figure 1B**) and core/helper lipid-free $LNP_{ssPalmE}$ (ssPalmE:Chol = 70:30 plus 3 mol% of DMG-PEG_{2000}) were compared using the plasma level of the FVII protein as an index at 24 hr after the administration at the indicated dose. The core/helper lipid-free $LNP_{ssPalmE}$ exhibited more potent gene knockdown efficiency.

Supplemental Figure S4



Supplemental Figure S4

Hemolysis and *in vivo* gene knockdown activity of LNPs prepared with ssPalmE-P4 series

A. Erythrocytes were incubated with LNPs prepared with the ssPalmE-P4 series of molecules at various pH values for 1 h. The absorbance of the hemoglobin that had leaked into the supernatant was measured. The values are represented as relative values of the positive control, obtained by triton X-100 treatment. At both pH of 6.5 and 7.4, ssPalmE-P4-C2 represented the maximum hemolysis activity. The rank-order of the hemolysis activity is completely matched to the pKa values determined in **Figure 2**.

B. The gene knockdown activities of LNPs were measured at 24 h after the i.v. administration at indicated dose. The maximum gene knockdown activity was exhibited in $LNP_{ssPalmE\text{-}P4\text{-}C2}$. This result was, in turn, consistent with the highest pKa value and potent hemolysis activity.

Supplemental Table S1

siRNA sequences used in the present study

siFVII (2'-OH)	Sense	5'-GGAUCAUCUCAAGUCUUACdTdT-3'
	Antisense	5'-GUAAGACUUGAGAUGAUCCdTdT-3'
siFVII (2'-F)	Sense	5'-GGAucAucucAAGucuuAcdT*dT-3'
	Antisense	5'-GuAAGAcuuGAGAuGAuccdT*dT-3'

dT: deoxytymidine

*: phosphorothioate linkage

Capital letter: native (2'-OH) ribonucleotides

Small letter: 2'-Fluoro-modified nucleotides

Supplemental Table S2

Physicochemical characters of LNPs used in Figure 1

	ssPalmM	ssPalmA	ssPalmE
Size (nm)	166±9	201±15	195±8
PdI	0.12±0.02	0.16±0.04	0.13±0.00
ζ-potential (mV)	-4.0±1.6	-10.5±1.9	-11±2.3
Encapsulation (%)	92±2	92±3	90±2