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Supplemental Information

Hydrophobicity of Lipid-Conjugated siRNAs Predicts Productive Loading to Small Extracellular Vesicles

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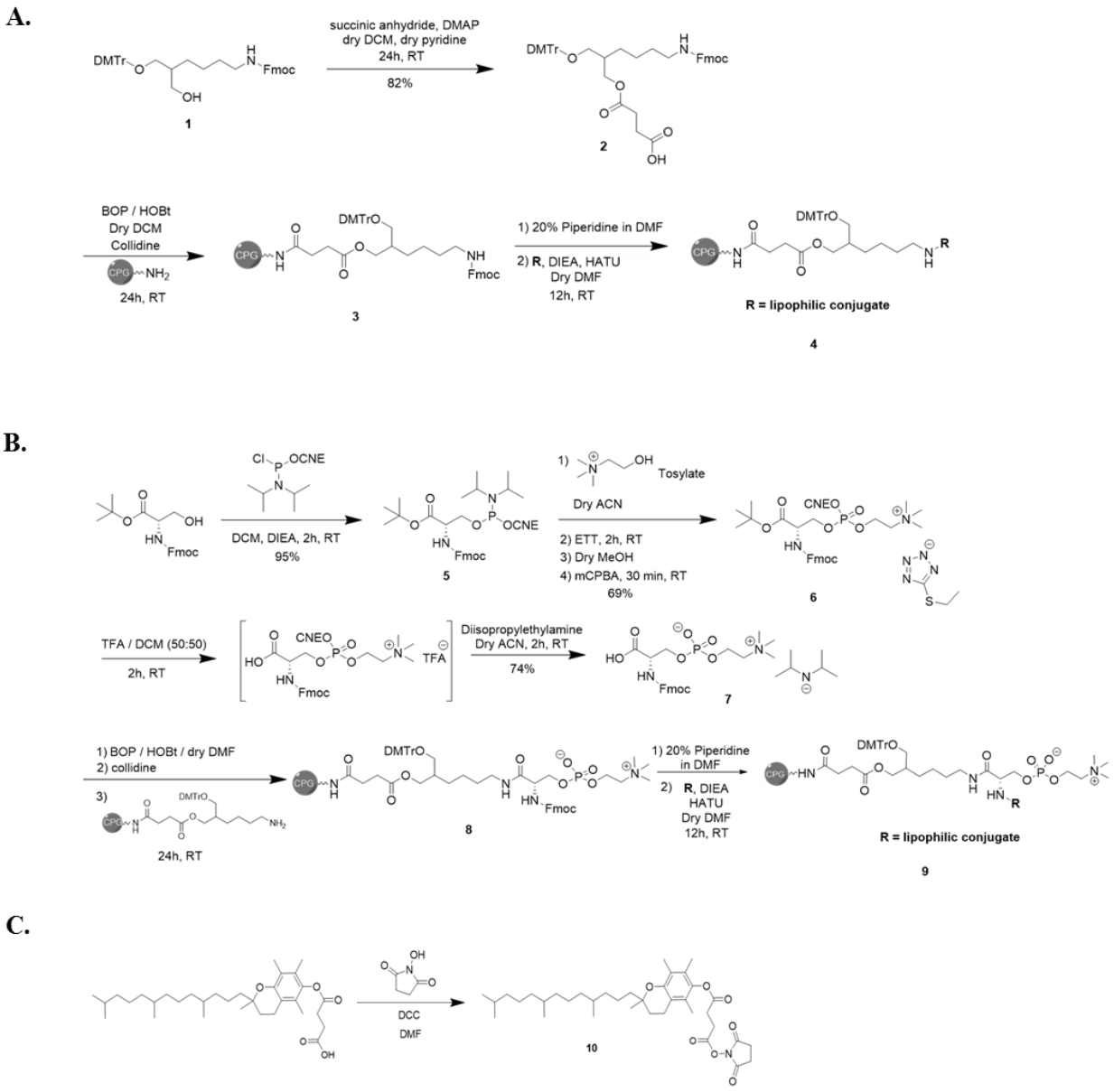


Scheme S1. Synthetic route of lipophilic compounds used for the synthesis of lipid conjugated siRNAs, Related to Figure 2. (A) Synthesis of solid supports conjugated with various lipophilic moieties (B) Synthesis of solid supports conjugated with various phosphocholine lipophilic moieties attached though a C7 linker. (C) Synthesis of NHS-α-tocopheryl succinate compound for post-synthetic conjugation.

(**A**) C7 linker (90% purity) **1** (13.00 g, 19.35 mmol, 1.0 equiv.), 4-dimethylaminopyridine (DMAP) (cat.) and succinic anhydride (2.68 g, 27.09 mmol, 1.4 equiv.) were dissolved in 120 mL of dry dichloromethane (DCM) and 34 mL of dry pyridine. The mixture was stirred 24h at room temperature and then washed with 300 mL of 10% citric acid. The organic layer was then washed with water and brine and dried over magnesium sulfate. The solvent was evaporated under pressure. A column chromatography on silica gel was performed using a gradient of methanol in a mixture of DCM:pyridine 99:1 from 0 to 10% to obtain **2** (12.24 g, 15.87 mmol, 82%).

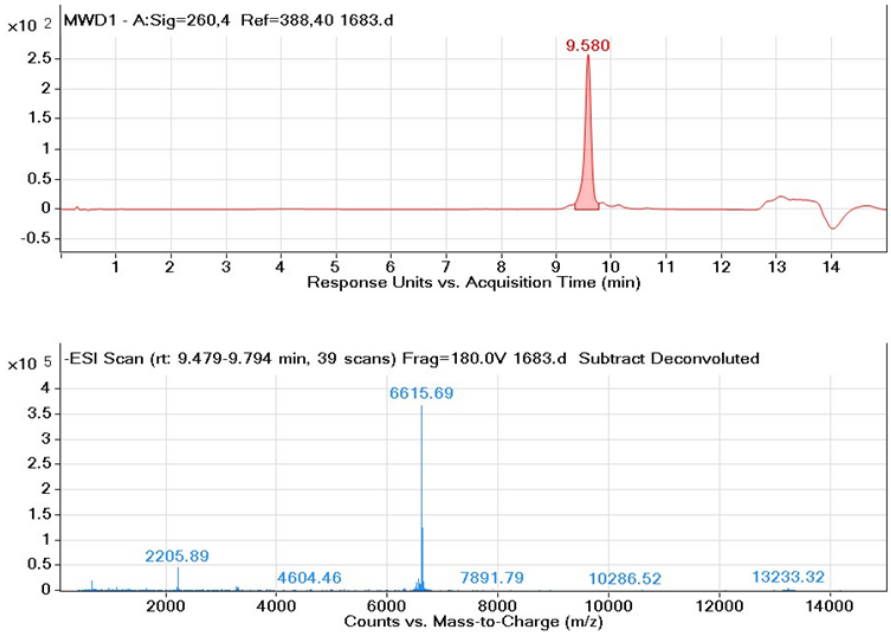
Compound **2** (5.72 g, 7.35 mmol, 2.2 equiv.), (Benzotriazol-1-yloxy) tris (dimethylamino) phosphonium hexafluorophosphate (BOP) (4.43 g, 10.02 mmol, 3.0 equiv.) and 1-Hydroxybenzotriazole (HOBt) (1.53 g, 10.02 mmol, 3.0 equiv.) were dissolved in 100 mL of dry DCM. The mixture was stirred few minutes and 2,4,6-collidine (2.61 mL, 20.04 mmol, 6.0 equiv.) was added. The amino controlled pore glass (CPG) (22.00 g, 3.34 mmol, 152 µmol/g) was added after treated with 250 mL of 3% TFA in DCM at room temperature for 4h, filtrated and washed first with TEA:diisopropylethylamine 9:1 (250 mL) and then with DCM and ether. The mixture was stirred mechanically 24h at room temperature. The CPG was washed with DCM, acetonitrile (ACN) and ether and dried under pressure. The CPG was then capped with 16% N-methylimidazole in tetrahydrofuran (THF) (CAP A) and acetic anhydride:pyridine:THF (1:2:2, v/v/v) (CAP B) (1:1, v/v) for 1h and was washed with DCM, ACN and ether and dried under vacuum. **3** is obtained with a loading of 75 µmol/g.
The CPG **3** (1.00 equiv.) was treated with a solution of 20% piperidine in dry dimethylformamide (DMF) (150 mL) two times 15 minutes, washed with DCM, ACN and ether and dried under pressure.
The selected lipid R (6.00 equiv.) was dissolved in 150 mL of dry DMF. 1-[Bis(dimethylamino)methylene]-1H-1,2,3-triazolo[4,5-b]pyridinium 3-oxid hexafluorophosphate (HATU) (2.00 equiv.) and diisopropylethylamine (DIEA) (8.00 equiv.) were added and the solution was added to the deprotected CPG. The mixture was stirred overnight under mechanical stirring at room temperature. The CPG was washed with DCM, ACN and ether and dried under pressure. The CPG was then capped with 16% N-methylimidazole in THF (CAP A) and acetic anhydride:pyridine:THF (1:2:2, v/v/v) (CAP B) (1:1, v/v) for 1h and was washed with DCM, ACN and ether and dried under vacuum. The lipid functionalized solid supports **4** were obtained with a loading of 55 µmol/g.

(**B**) Fmoc-L-serine-tBu (2.00 g, 5.21 mmol, 1.0 equiv.) was first dried by co-evaporation with toluene. Dry DCM (15 mL) and diisopropylethylamine (DIPEA) (1.54 mL, 8.86 mmol, 1.7 equiv.) were added under argon and 2´-cyanoethyl-N,N-diisopropylchlorophosphoramidite (1.60 g, 6.78 mmol, 1.3 equiv.) was added slowly via a syringe. The reaction mixture was stirred 2h at room temperature. After reaching completion, the reaction mixture was quenched with methanol and was washed with a solution of sodium bicarbonate and brine. The aqueous phase was extracted with DCM. The organic phase was dried on magnesium sulfate, filtrated and evaporated under vacuum. The crude mixture was then purified by column chromatography on silica gel using ethyl acetate/Hexane (8/2) with 1% pyridine as eluent, to afford **5** as a white solid (2.90 g, 4.97 mmol, 95%).
Compound **5** (2.90 g, 5.39 mmol, 1.0 equiv.) was dried with dry toluene and dry ACN. Choline p-toluenesulfonate (1.63 g, 5.93 mmol, 1.1 equiv.) was dried with toluene and dissolved in dry ACN (46 mL). This mixture was added to compound **5** through a cannula. 5-(Ethylthio)-1*H*-tetrazole (ETT) (0.25 M in ACN) (21.6 mL, 5.39 mmol, 1.0 equiv.) was added slowly with a syringe. The mixture was stirred 2h at room temperature. After reaching completion, the reaction mixture was quenched with methanol. Meta-chloroperoxybenzoic acid (mCPBA) (1.86 g, 10.78 mmol, 2.0 equiv.) was added by portion to the mixture. After 30 min of stirring, the mixture was reduced under vacuum. The crude was then purified by column chromatography on silica gel using a gradient of Methanol in DCM (0-30%) as eluent, to obtain **6** as a mixture of tetrazolium (major counter anion) and tosylate (less than 5%) salts (2.70 g, 3.69 mmol, yield 69%).
Compound **6** (2.30 g, 3.15 mmol, 1.0 equiv.) was dissolved in 60 mL of (1:1) solution of trifluoroacetic acid (TFA):dry DCM. Triisopropylsilane (2.39 mL, 11.66 mmol, 3.7 equiv.) was added and the mixture was stirred at room temperature for 2h. The solvent and TFA were evaporated and the residue was purified by reverse phase HPLC (C_{18}, Buffer A = Water, Buffer B = ACN, Gradient = 5-65% of B in 12 min, T = 45°C). The ACN was removed under vacuum and the aqueous solution was freeze-dried. The lyophilized powder was dissolved in 10% diisopropylamine (14 mL) in ACN (140 mL) and the mixture was stirred at room temperature for 2h. The solvent was evaporated under vacuum and the crude was purified by reverse phase HPLC (C_{18}, Buffer A = Water, Buffer B = ACN, Gradient = 5-65% of B in 12 min, T = 45°C). The ACN was removed under vacuum and the aqueous solution was freeze-dried to afford **7** as diisopropylammonium salt (1.38 g, 2.32 mmol, yield 74% over two steps).
Compound **7** (1.00 g, 1.69 mmol, 4.75 equiv.) was dissolved in dry DMF (100 mL). (Benzotriazol-1-yloxy)tris(dimethylamino)phosphonium hexafluorophosphate (BOP) (0.59 g, 1.34 mmol, 3.76 equiv.) and hydroxybenzotriazol (HOBt) (0.21 g, 1.34 mmol, 3.76 equiv.) were added and stirred until the solution went clear. 2,4,6-collidine (560 µL, 4.32 mmol, 12.42 equiv.) was added followed by **3** deprotected with 20% piperidine in DMF (6.55 g, loading of 55 µmol/g, 360 µmol, 1.00 equiv.) and the suspension was mixed overnight on a rotary mixer. The CPG was filtered off and washed with DCM, ACN and ether and dried under vacuum. The CPG was capped with 16% N-methylimidazole in THF (CAP A) and acetic anhydride:pyridine:THF (1:2:2, v/v/v) (CAP B) (1:1, v/v) for 1h and was washed with DCM, ACN and ether and dried under vacuum.

CPG **8** (6.00 g, 330 µmol, 1.0 equiv.) was first treated with 20% piperidine in dry DMF for 15 minutes. This procedure was repeated twice to ensure complete deprotection of the Fmoc group. The amine-bearing CPG was filtered off and washed successively with DCM, ACN and ether and dried under vacuum. Then the CPG was mixed with a mixture of the selected lipid R (6.0 equiv.), HATU (2.0 equiv.) and DIEA (8.0 equiv.) in dry DMF. The suspension was mixed on a rotary mixer for 24h. The CPG was then filtered off and washed with DCM, ACN and ether and dried under vacuum. The CPG was capped with 16% N-methylimidazole in THF (CAP A) and acetic anhydride:pyridine:THF (1:2:2, v/v/v) (CAP B) (1:1, v/v) during 15 min and was washed with DCM, ACN and ether and dried under vacuum. The PC lipid functionalized solid supports **9** were obtained with a loading of 55 µmol/g.

(**C**) α-tocopheryl succinate (0.5 g, 0.94 mmol, 1.0 equiv.), N-hydroxysuccinimide (0.21 g, 1.88 mmol, 2.0 equiv.) and dicyclohexylcarbodiimide (DCC) (0.39 g, 1.88 mmo, 2.0 equiv.) were dissolved in 25 mL of anhydrous DMF. The mixture was stirred overnight at room temperature. The dicyclohexyl urea was filtrated and the filtrate was evaporated under pressure. The product **10** was isolated by precipitation with methanol (0.47 g, 0.75 mmol, 80%).

A.



B.

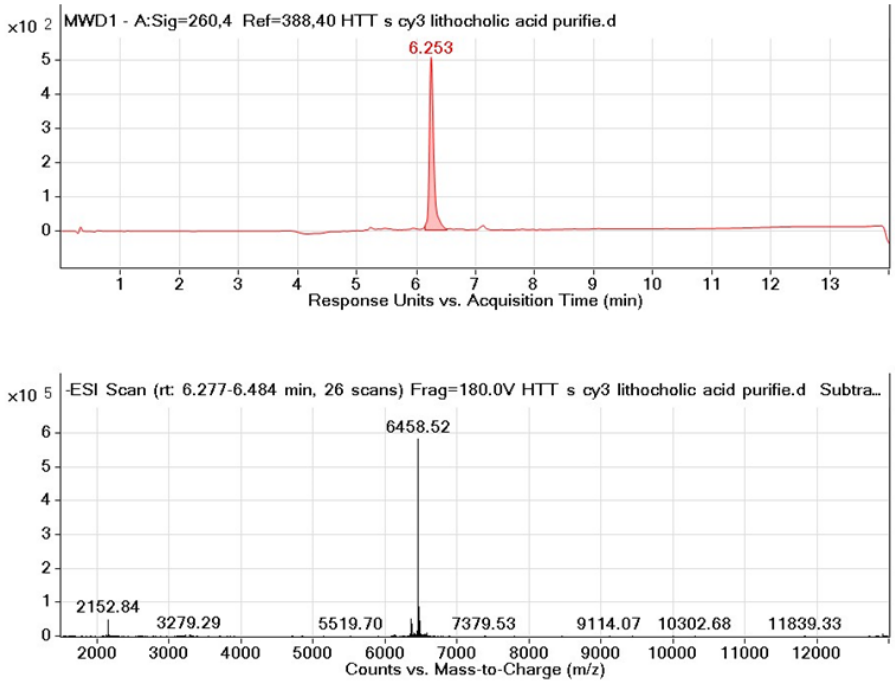


Figure S1. LC-MS characterization of purified oligonucleotides, Related to Figure 2. (A) hsiRNA^{HTT} antisense strand. (B) Cy3-hsiRNA^{HTT} sense strand conjugated with PC-Lithocholic acid.

Target	Name	Strand	Sequence 5'-3'
Huntingtin mRNA	hsiRNA^{Htt}	Sense	Cy3-(fC)#(mA)#(fG)(mU)(fA)(mA)(fA)(mG)(fA)(mG)(fA)(mU)(fU)#(mA)#(fA)-conjugate
		Antisense	E-VP-(mU)#(fU)#(mA)(fA)(mU)(fC)(mU)(fC)(mU)(fU)(mU)(fA)(mC)#(fU)#(mG)#(fA)#(mU)#(fA)#(mU)#(fA)

m = 2'-*O*-methyl ; f = 2'-fluoro ; # = phosphorothioate linkage ; hsiRNA = hydrophobically modified small interfering RNA ; E-VP = E-Vinyl Phosphonate

Table S1. hsiRNA sequences used in this study, Related to Figure 1.

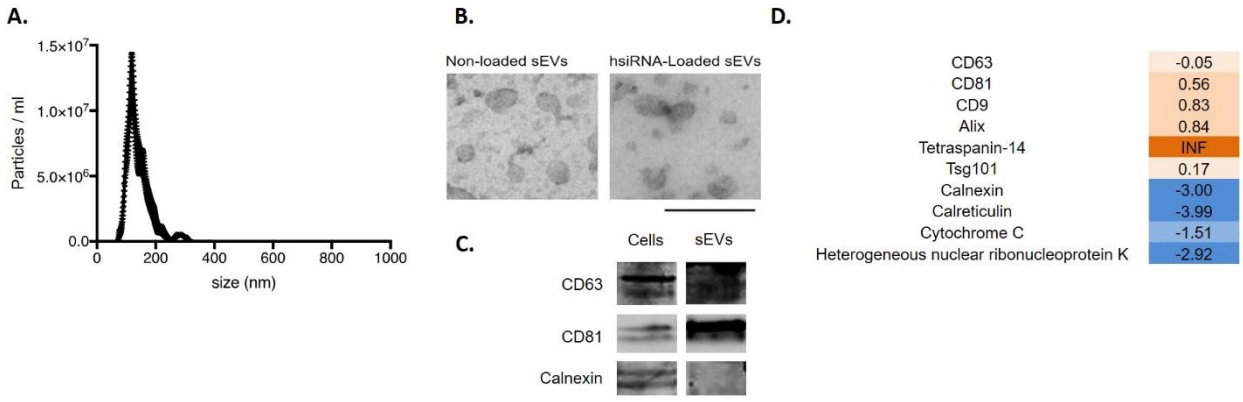


Figure S2. Characterization of umbilical cord, Wharton's jelly derived sEVs, Related to Figure 3. Umbilical cord, Wharton's jelly derived mesenchymal stem cells were expanded to passage 9 at 3600 cm^2, medium changed to serum-free RPMI for 24 hours, and sEVs purified from conditioned media via differential ultracentrifugation. (A**)** Nanoparticle Tracking Analysis of 100,000 g fraction from differential ultracentrifugation protocol (e.g. small EVs). N=11, mean ± SEM (B) Transmission Electron Microscopy image of unloaded and loaded sEVs, size bar shows 500 nm. (C) Western blot of positive and negative sEV marker proteins. (D) Protein enrichment (logarithmic) in sEVs *versus* cells as detected by LC-MS/MS. INF=infinite (detected in sEV fraction but not detected in cells)

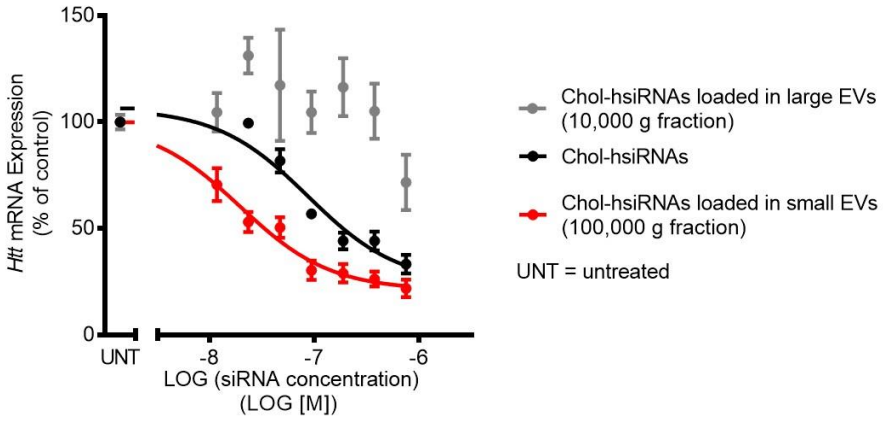


Figure S3. Silencing activities of cholesterol conjugated hsiRNA-loaded sEVs using 10,000 and 100,000 g pellet fractions and cholesterol conjugated hsiRNA, Related to Figure 3. *Htt* mRNA levels in primary mouse neurons incubated with increasing concentrations of cholesterol conjugated hsiRNA^{Htt}-loaded small EVs (100,000 g fraction), large EVs (10,000 g fraction) or cholesterol conjugated hsiRNA^{Htt} for one week. *Htt* mRNA levels were normalized to Hprt (Hypoxanthine-guanine phosphoribosyl transferase), and presented as percent of untreated control (n=3, mean ± SEM). UNT, untreated

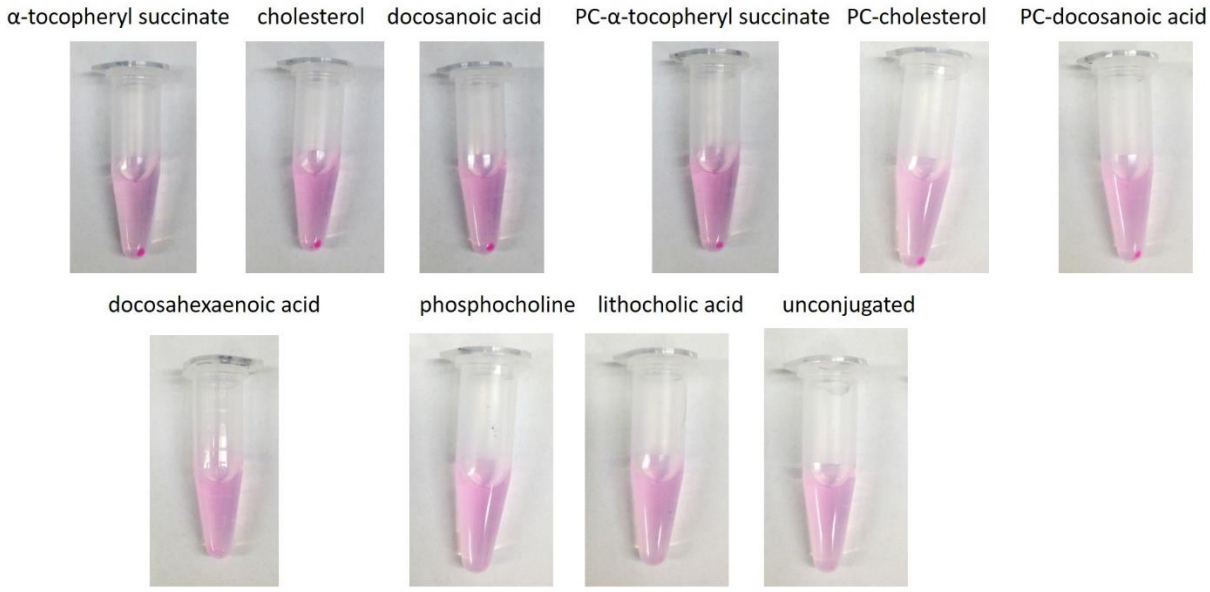


Figure S4. Pictures of sEVs loaded with lipid-conjugated hsiRNAs after ultracentrifugation, Related to Figure 3.

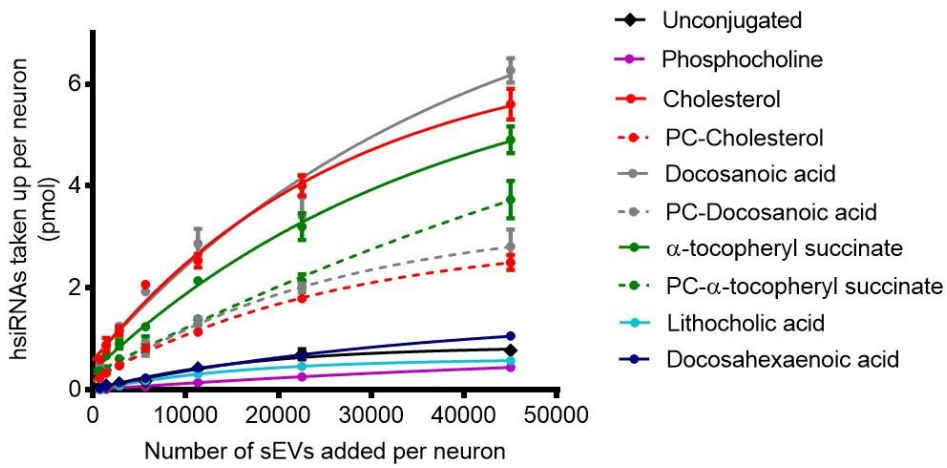


Figure S5. Uptake efficiency of hsiRNA loaded sEVs by neurons, Related to Figure 4. hsiRNAs levels in neurons were quantified using PNA hybridization assay after incubation of neurons with increasing amounts of loaded sEVs for one week. (n=3, mean ± SEM).