Electronic Supplementary Material (ESI) for Organic & Biomolecular Chemistry.

Efficacious redox-responsive gene delivery in serum by ferrocenylated monomeric and dimeric cationic cholesterols

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Materials and Methods. All reagents, solvents and chemicals used in this study were of the highest purity available. The solvents were dried prior to use. Column chromatography was performed using 60-120 mesh silica gel or neutral alumina. NMR spectra were recorded using a bruker spectrometer (400 MHz for ^{1}H and 100 MHz for ^{13}C NMR) spectrometer. The chemical shifts (δ) are reported in ppm downfield from the internal standard; TMS, for ^{1}H-NMR. Mass spectra were recorded on a MicroMass ESI-TOF spectrometer. Lipids were synthesized as described below.

Cholest-5-en-3β-oxyethan-*N*,*N*-dimethyl amine (Chol $OCH_2CH_2NMe_2$) was synthesized from cholesterol as described earlier.[1]
The intermediates **1-8** were synthesized according to previously reported procedures.[2-4]

General procedure for the synthesis of ω-Bromo-alkanyl-1-monoacyl ferrocenes (**1-2**). A solution of 6-bromohexanoic acid or 11-bromoundecanoic acid (10 mmol) in 4 mL of $SOCl_2$ was stirred for 1 day. The solution was then concentrated *in vacuo* to give a crude product of corresponding ω-bromoalkanoyl chloride which was dissolved in 20 mL of dry CH_2Cl_2 and was added dropwise to a mixture of ferrocene (12 mmol) and anhydrous $AlCl_3$ (13 mmol) in 50 mL of dry CH_2Cl_2 over 1 h under argon. After stirring for 12 h, the solution was poured into ice-water saturated with NaCl. The organic layer was washed with brine, dried with $MgSO_4$ and concentrated *in vacuo*. The residue was purified by column chromatography over silica gel using hexane/ethyl acetate to afford the pure compounds in 55-60% yield.

1-(6-Bromohexanoyl) ferrocene[2] **(1)**: ^{1}H NMR (400 MHz, $CDCl_3$) δ 4.78 (s, 2H), 4.50 (s, 2H), 4.20 (s, 5H), 3.45 (t, 2H, *J* = 6.8 Hz), 2.73 (t, 2H, *J* =7.2 Hz). ESI-MS *m/z* Calcd. for $C_{16}H_{19}BrFeONa$ 384.99, found 385.00 (M+Na).

1-(11-Bromoundecanoyl) ferrocene[3] **(2)**: ^{1}H NMR (400 MHz, $CDCl_3$): δ 4.78 (d, 2H, *J* = 4.8 Hz), 4.50 (d, 2H, *J* = 4.8 Hz), 4.10 (s, 5H), 3.40 (t, 2H, *J* = 6.9 Hz), 2.62 (t, 2H, *J* = 7.5 Hz), 1.84 (m, 2H, *J* = 7.2 Hz), 1.68 (m, 2H) and 1.32 (m, 12H). ESI-MS (HRMS) *m/z* Calcd. for $C_{21}H_{29}BrFeONa$ 455.0649, found 455.0649 (M+Na).

General procedure for synthesis of ω-Bromo-alkanoyl-1,1'-diacylferrocenes (5-6): Same procedure as described above for the monoacyl ferrocenes was followed except that ferrocene (10 mmol) dissolved in dry CH_2Cl_2 (20 ml) was added dropwise to a stirred solution of ω-bromoalkanoyl chloride (24 mmol) and anhydrous $AlCl_3$ (26 mmol) in 50 mL of dry CH_2Cl_2 over a period of 3 h. The yields ranged from 45-55%.

1,1'-Bis(6-bromohexanoyl)ferrocene[4] **(5)**: ^{1}H NMR (400 MHz, $CDCl_3$): δ 4.76 (s, 4 H), 4.50 (s,4 H), 3.44 (t, 4 H, *J* = 6.4 Hz), 2.66 (t, 4 H, *J* = 7.2 Hz), 1.97-1.41 (m, 12 H). ESI-MS *m/z* Calcd. for $C_{22}H_{28}Br_2FeO_2Na$ 562.86, found 562.86 (M+Na).

1,1'-Bis(11- bromoundecanoyl)ferrocene[4] **(6)**: ^{1}H NMR (400 MHz, $CDCl_3$): 4.77 (s, 4 H), 4.48 (s,4 H), 3.41 (t, 4 H, *J*= 6.9 Hz), 2.64 (t, 4 H, *J* = 7.4 Hz), 1.85 (m,4 H), 1.68-1.28 (m, 28 H). ESI-MS *m/z* Calcd. For $C_{32}H_{48}Br_2FeO_2Na$ 701.12, found 701.10 (M+Na).

Synthesis of 1-(ω-Bromoalkyl)ferrocenes (3-4) and 1,1'-Bis(ω-bromoalkyl) ferrocenes (7-8)
To a stirred mixture of anhydrous aluminium chloride (4 mmol) and sodium borohydride (8 mmol) in dry THF at 0 ˚C, a solution of **1-2 or 5-6** (1 mmol) in THF were added dropwise under Ar atmosphere. It was stirred for 4h and after which TLC showed end of reaction. The reaction mixture was then quenched by careful addition of 10 mL of ice-water. The reaction mixture was washed with water and brine, extracted using ethyl acetate. The organic layer was separated, dried with anhydrous $MgSO_4$, and concentrated *in vacuo*. The residue was purified by column chromatography over silica gel using hexane/ethyl acetate (20/1). The yields ranged from 85-90 % for (**3-4**) and 75-80 % for (**7-8**).

1-(6-Bromohexyl)ferrocene[3]**(3)**: ^{1}H NMR (400 MHz, $CDCl_3$) δ 4.10(s, 5H), 4.05(s, 4H), 3.41(t, 2H), 2.32(t, 2H), 1.86(t, 2H), 1.47(m, 4H), 1.34(m, 2H). HRMS *m/z* Calcd. for $C_{16}H_{21}BrFe$ 348.0176 (M^+), found 348.0179 (M+).

(11-Bromoundecyl) ferrocene[3] **(4)**: ^{1}H NMR (400 MHz, $CDCl_3$) δ 4.20 (m, 9H), 3.41(t, 2H), 2.24 (t, 2H), 1.86 (m, 2H), 1.45 (m, 4H), 1.28 (m, 12 H). HRMS *m/z* Calcd. for $C_{21}H_{31}BrFe$ 418.0959 (M^+), found 418.0955(M^+).

1,1'-Bis(6-bromohexy1)ferrocene[4] **(7)**: ^{1}H NMR (400 MHz, $CDCl_3$) δ 3.99 (s,4H), 3.98 (s,4H), 3.42 (t,4H, *J* = 6.8 Hz), 2.32 (t, 4 H, *J* = 7.5 Hz), 1.47 (m,4 H), 1.53-1.35 (m, 12 H). HRMS *m/z* Calcd. for $C_{22}H_{32}Br_2Fe$ (M^+) 512.1511, found 512.1510 (M^+).

1,1'-Bis(6-bromoundecyl)ferrocene[4] **(8)**: ^{1}H NMR (400 MHz, $CDCl_3$) δ 4.06 (bd, 8 H), 3.49 (t, 4 H, *J* = 6.9 Hz), 2.38 (t, 4 H, *J* = 7.7 Hz), 1.94 (m,4 H), 1-57-1.28 (m, 32 H). HRMS *m/z* Calcd. for $C_{32}H_{52}Br_2FeK$ 689.19, found 689.16 (M+K).

General Method for the Synthesis of the Ferrocenylated Lipids (CHM-C6F, CHM-C11F, CHD-C6F and CHD-C11F). A solution of cholest-5-en-3*β*-oxyethan-N,N-dimethylamine (0.2 mmol) was added to appropriate 1-(ω-bromoalkyl)ferrocenes **(3-4**, 0.22 mmol**)** or 1,1'-bis(ω-bromoalkyl) ferrocenes **(7-8**, 0.07 mmol) in dry MeOH-EtOAc (4 mL, v/v: 1/1). It was refluxed over a period of 4-6 days in a screw-top pressure tube, until TLC indicated the completion of reaction. After that, the reaction mixture was cooled and the solvent was evaporated to furnish a crude solid which was purified by column chromatography over neutral alumina using $CHCl_3$ to $CHCl_3$-MeOH. Yields varied from 40-50%.

CHM-C6F: ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.36 (br s, 1H), 4.14 (m, 9H), 3.94 (s, 4H), 3.56 (br t, 2H), 3.38 (s, 6H), 3.2 (m, 1H), 2.34 (t, 2H, *J* = 6.92 Hz), 2.12-0.9 (m, 48H), 0.68(s, 3H). ^{13}C NMR(100 MHz, $CDCl_3$) 139.6, 122.6, 79.9, 68.5, 68.1, 67.1, 61.9, 56.6, 56.1, 51.9, 50.0, 42.3, 39.7, 39.5, 38.8, 36.8, 36.7, 36.1, 35.8, 31.8, 31.0, 29.4, 29.0, 28.2, 28.0, 26.1, 24.2, 23.8, 22.8, 22.5, 21.0, 19.3, 18.6, 11.8 HRMS *m/z* Calcd. for $C_{47}H_{76}FeNO^+$ 726.5271, found 726.5277 (M^+). Anal. Calcd for $C_{47}H_{76}BrFeNO.H_2O$: C, 68.43; H, 9.53; N, 1.70; Found: C, 68.18; H, 9.80; N, 1.54

CHM-C11F: ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.36 (br s, 1H), 4.1 (m, 9H), 3.94 (s, 4H), 3.56 (br s, 2H), 3.39 (s, 6H), 3.2 (m,1H), 2.32 (m, 2H), 2.12-0.9 (m, 58H). 0.65(s, 3H). ^{13}C NMR(100 MHz, $CDCl_3$) 139.7, 122.5, 80.0, 76.7, 68.8, 68.3, 67.2, 66, 63.2, 61.9, 56.7, 56.1, 51.9, 50.0, 42.3, 39.7, 39.5, 38.8, 36.9, 36.1, 35.7, 31.9, 31, 29.6, 29.4, 29.1, 28.1, 28, 26.2, 24.2, 23.8, 22.8, 22.5, 21.0, 19.3, 18.7,11.8 HRMS *m/z* Calcd. for $C_{52}H_{86}FeNO^+$ 796.6059 found 796.6056 (M^+). Anal. Calcd for $C_{52}H_{86}BrFeNO$: C, 71.22; H, 9.88; N, 1.60. Found: C, 71.26; H, 9.59; N, 1.85

CHD-C6F: ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.36 (br s, 2H), 4.1 (m, 8H), 4.0-3.8 (m, 12H), 3.42 (s, 12H), 3.21 (m, 2H), 2.3-0.85 (m, 100H), 0.68 (s, 6H). ^{13}C NMR(100 MHz, $CDCl_3$) 139.7, 122.5, 80.0, 68.8, 67.4, 66.2, 63.3, 61.9, 56.7, 56.1, 51.9, 50.0, 42.3, 39.7, 39.5, 38.7, 36.9 36.7, 36.1,35.7, 30.8, 29.1, 28.9, 28.2, 28.1, 26.0, 24.2, 23.8, 22.9, 22.8, 22.5, 21.0, 19.3, 18.7, 11.8 HRMS *m/z* Calcd. for $C_{84}H_{142}FeN_2O_2^{2+}$ 1267.041, found 633.5202 (M^{2+}/2). Anal. Calcd for $C_{84}H_{146}Br_2FeN_2O_4.2H_2O$: C, 68.93; H, 10.05; N, 1.91. Found: C, 68.70; H, 10.17; N, 1.85

CHD-C11F: ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.36 (br s, 2H), 4.05-3.9 (m, 16H), 3.7 (bs, 4H), 3.45 (br s, 12H), 3.2 (m, 2H), 2.38-0.8 (bm, 120Hs), 0.65(s, 6H). ^{13}C NMR(100 MHz, $CDCl_3$) 139.6, 122.3, 80.0, 69.0, 67.9, 65.8, 63.1, 61.8, 56.6, 56.0, 51.8, 49.9, 42.2, 39.4, 38.6, 36.6, 35.6, 31.7, 31.0, 29.5, 29.4, 29.3, 29.1, 28.1, 28.0, 27.9, 26.1, 24.1, 23.7, 22.7, 22.4, 20.9, 19.2, 18.6, 11.7 HRMS *m/z* Calcd. for $C_{94}H_{162}FeN_2O_2^{2+}$ 1407.1975, found 703.5992 (M^{2+}/2). Anal. Calcd. for $C_{94}H_{162}Br_2FeN_2O_2.H_2O$: C, 70.78; H, 10.43; N, 1.76, found: C, 70.55; H, 10.59, N, 1.90

References:

(1) A. Bajaj, P. Kondiah and S. Bhattacharya, *J. Med. Chem.*, 2007, **50**, 2432.

(2) K. C. Majumdar, S. Chakravorty, N. Pal and R. K. Sinha, *Tetrahedron*, 2009, **65**, 7998.

(3) Y. Han, K. Cheng, K. A. Simon, Y. Lan, P. Sejwal and Y. Y. Luk, *J. Am. Chem. Soc.*, 2006, **128**, 13913.

(4) J. Bhatt, B. M. Fung and K. M. Nicholas, *Liquid Crystals*, 1992, **12**, 263.

^{1}H-NMR and ^{13}C-NMR spectra of final lipid molecules.

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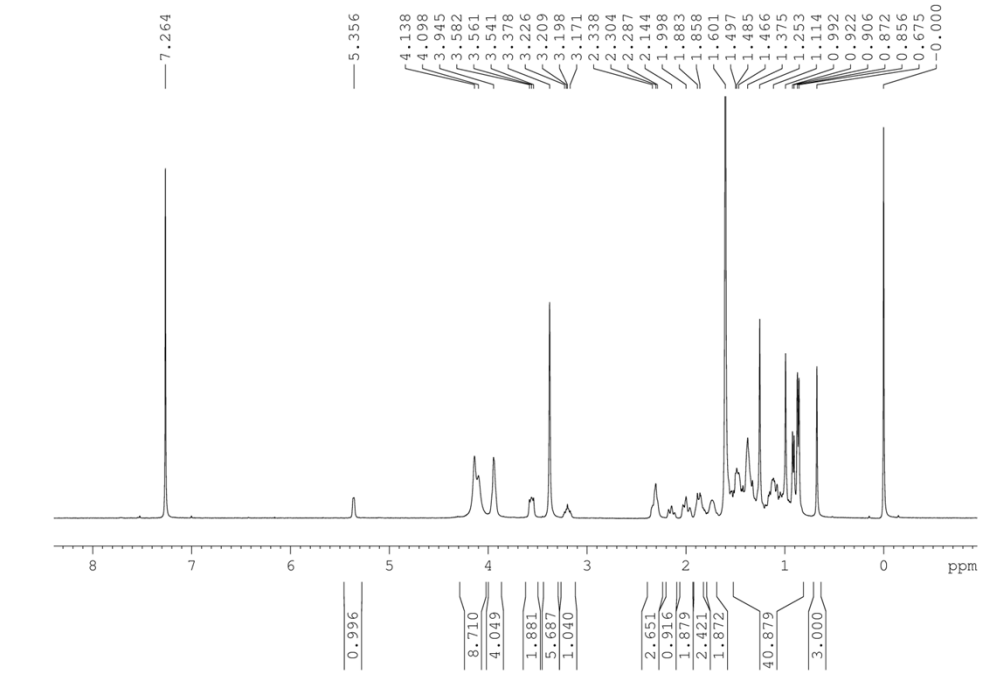

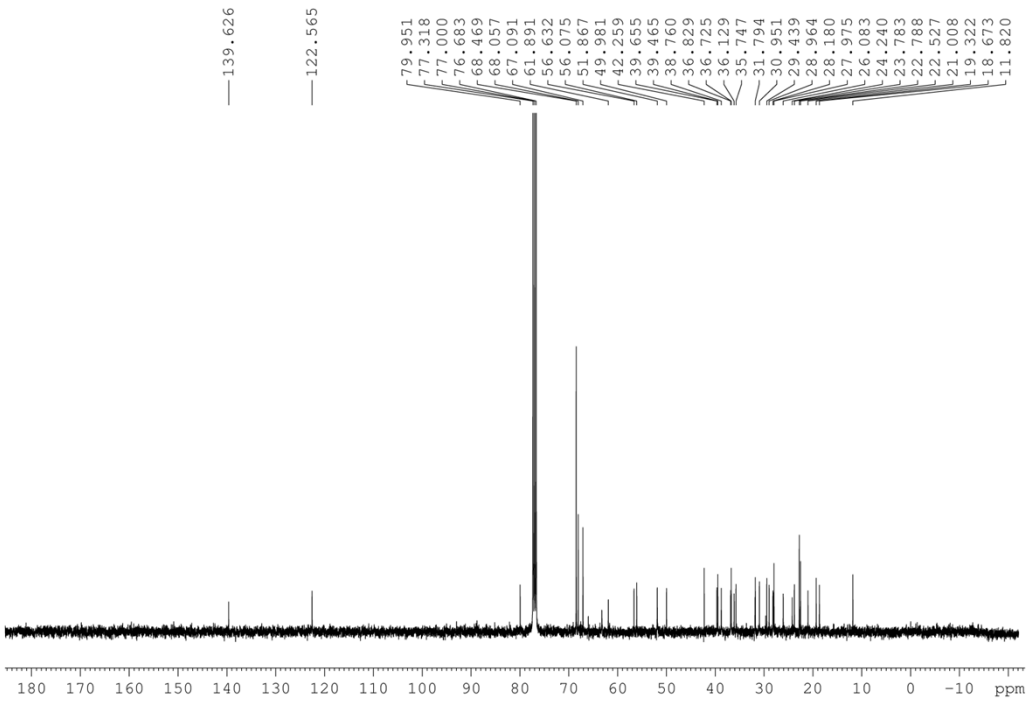

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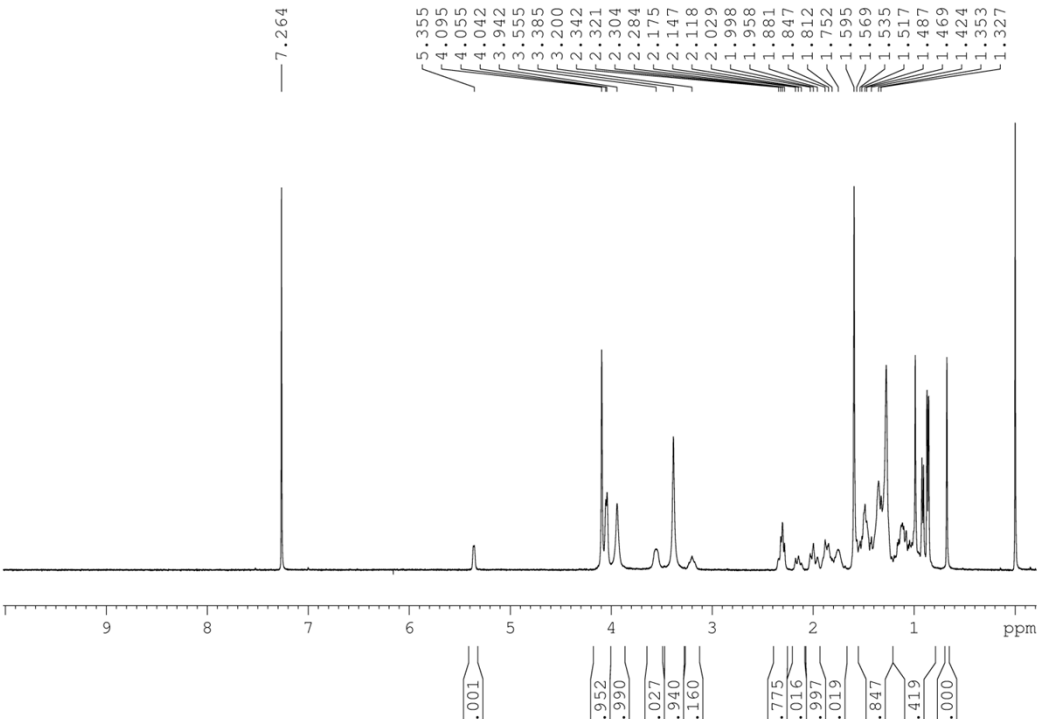
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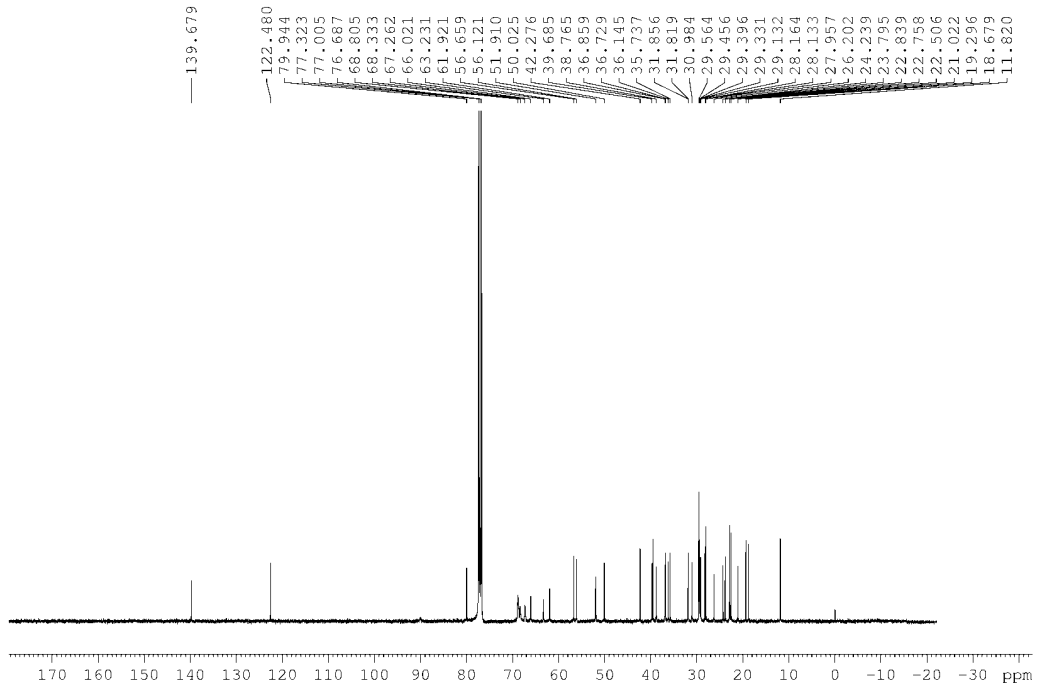
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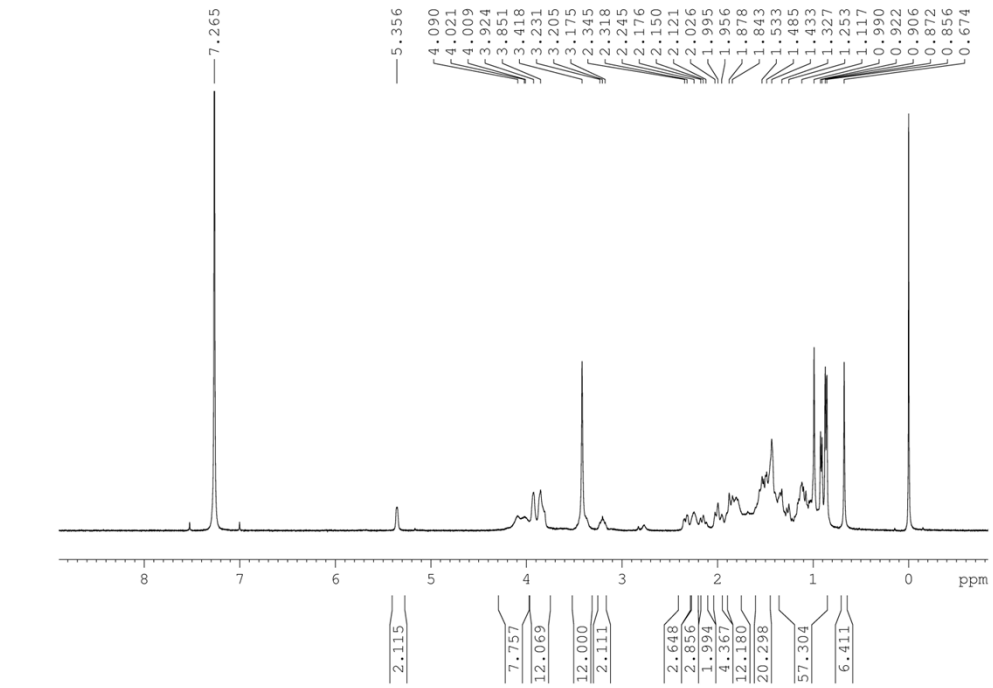
(3) CHD-C6F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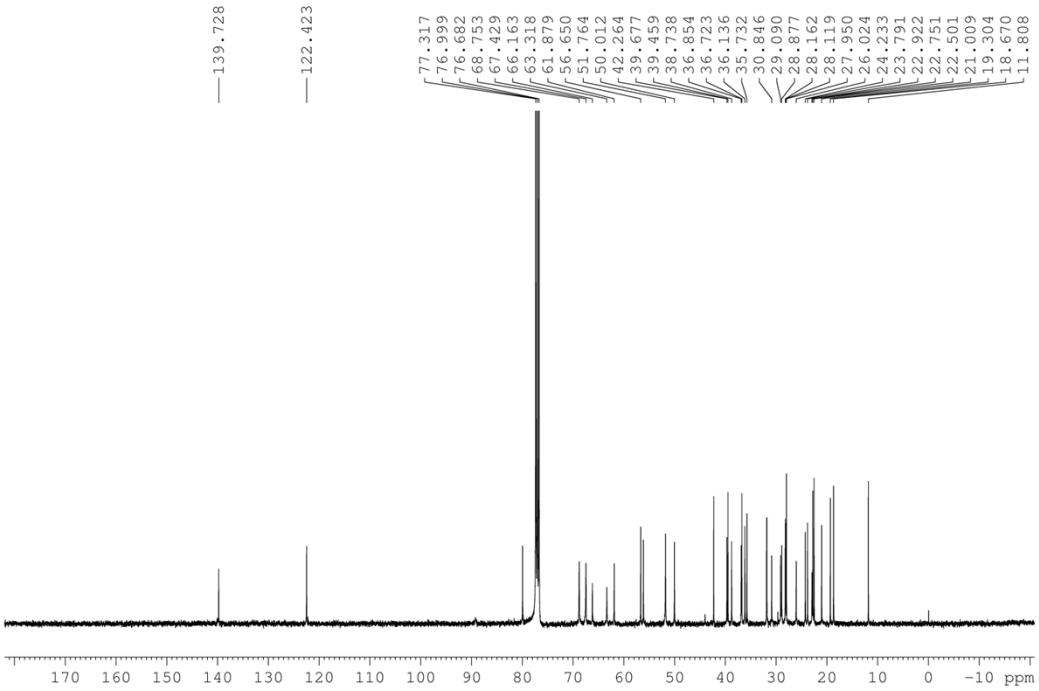
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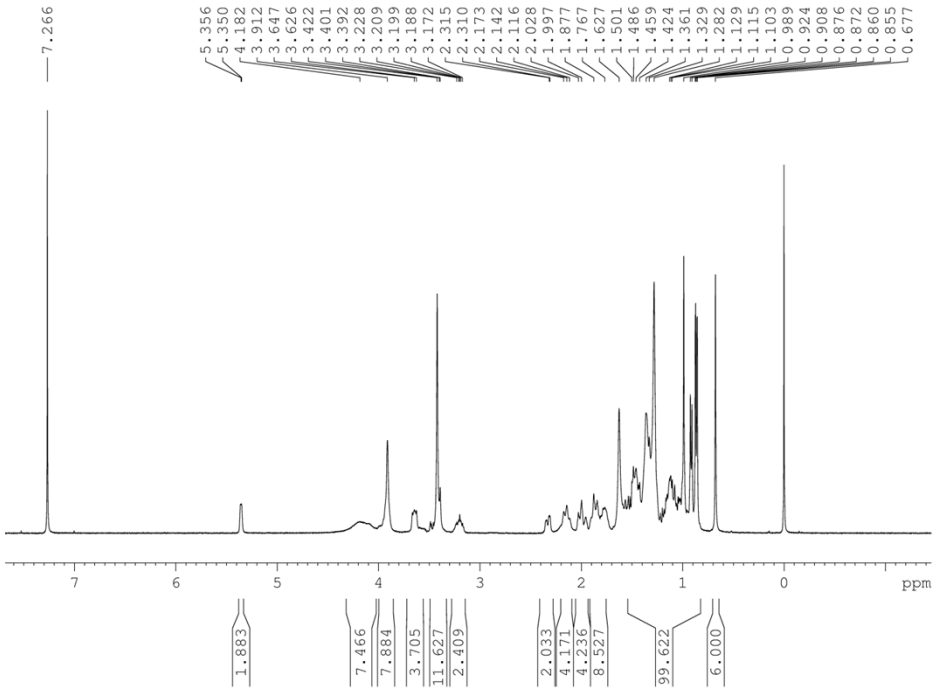
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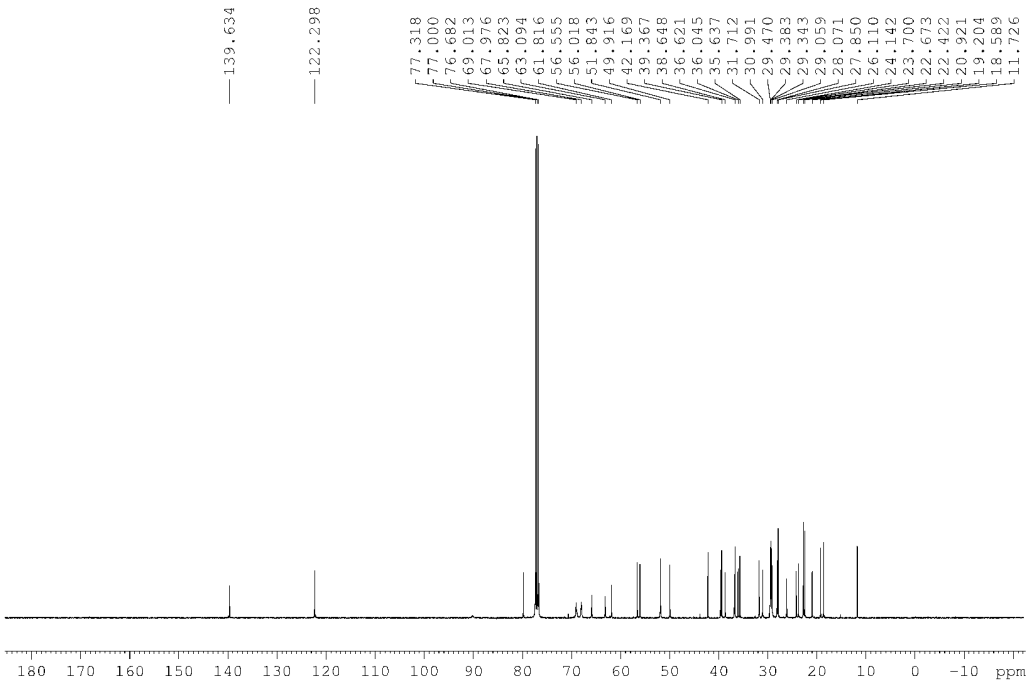
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40
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ppm

Table S1. Average Hydrodynamic Diameters and Zeta potentials of the redox-active cationic cholesterol/DOPE aggregates.[a]

Mixed Liposomes	Avg. hydrodynamic diameter (nm)		PDI		Zeta potential (mV)	
	Red	Ox	Red	Ox	Red	Ox
DOPE/CHM-C6F	140 ± 10	186 ± 20	0.12	0.20	30 ± 0.5	36.5 ± 2.5
DOPE/CHM-11F	152 ± 10	205 ± 25	0.1	0.22	28.5 ± 0.5	38 ± 2.3
DOPE/CHD-C6F	175 ± 17	232 ± 35	0.11	0.27	42 ± 1	49.5 ± 3
DOPE/CHD-C11F	207 ± 25	270 ± 30	0.17	0.27	39.5 ± 2.2	51.2 ± 3

[a] Hydrodynamic diameters and zeta potentials of co-liposomes of each redox-active cationic cholesterol/DOPE at the transfection optimized ratio prepared in 10 mM HEPES buffer (pH = 7.4) and cationic lipid concentration of 0.1 mM. Each value is a mean ± S.D of five independent measurements. PDI: Polydispersity index.

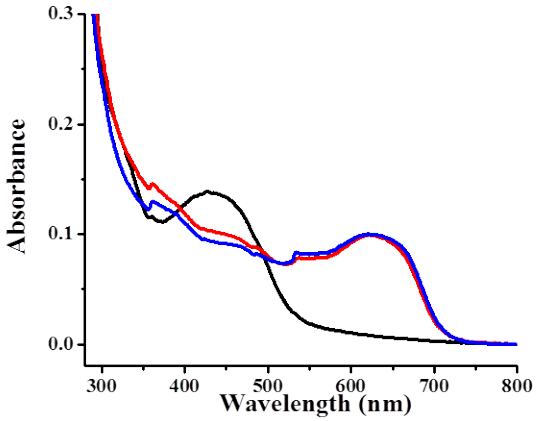

Fig. S1 Representative UV-visible absorbance spectra of CHM-C6F co-liposomal suspensions in their reduced state (black line) and after oxidation; freshly prepared (red line), after 96 h (blue line).

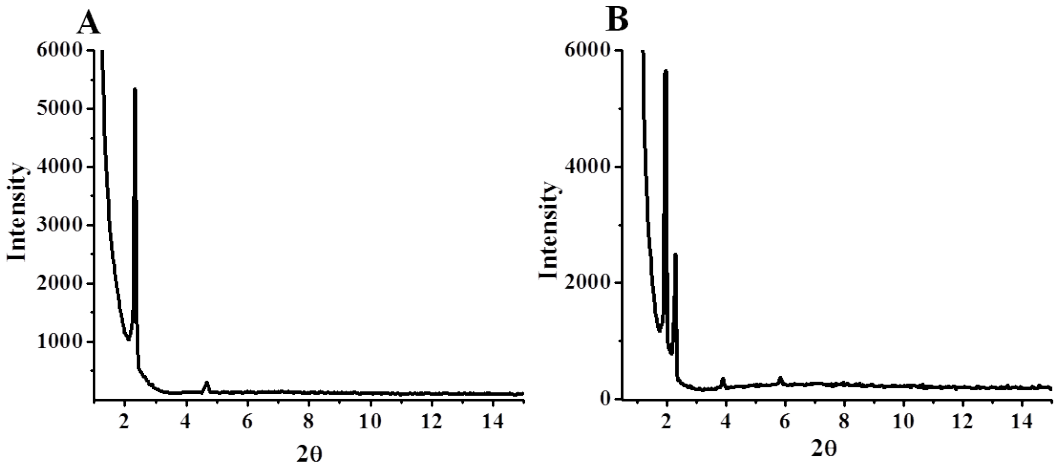

Fig. S2 X-Ray diffraction spectra of representative co-liposomes of CHM-C6F/DOPE and CHD-C6F/DOPE.

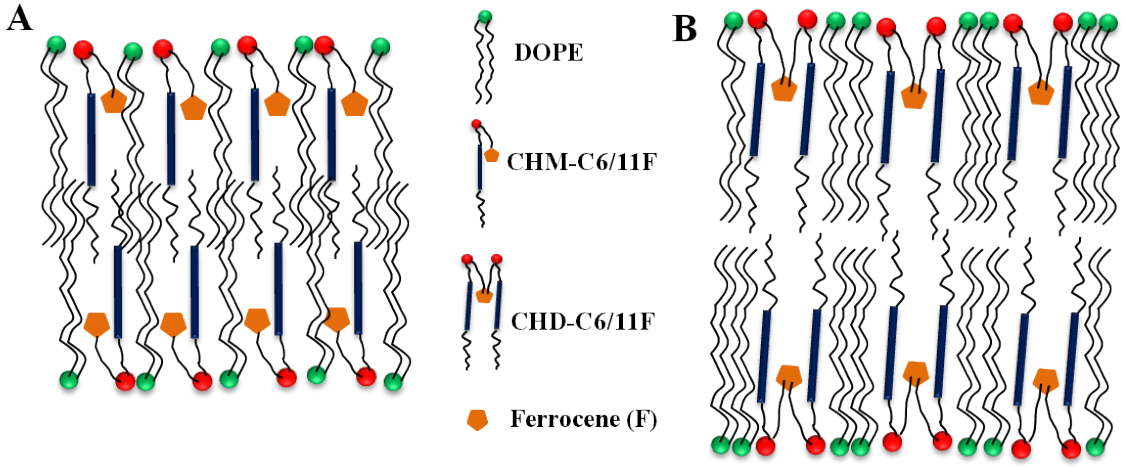

Fig. S3 The schematic representation of lipid molecular packing in bilayer arrangements of redox-active cationic monomeric (A) and gemini (B) lipids.

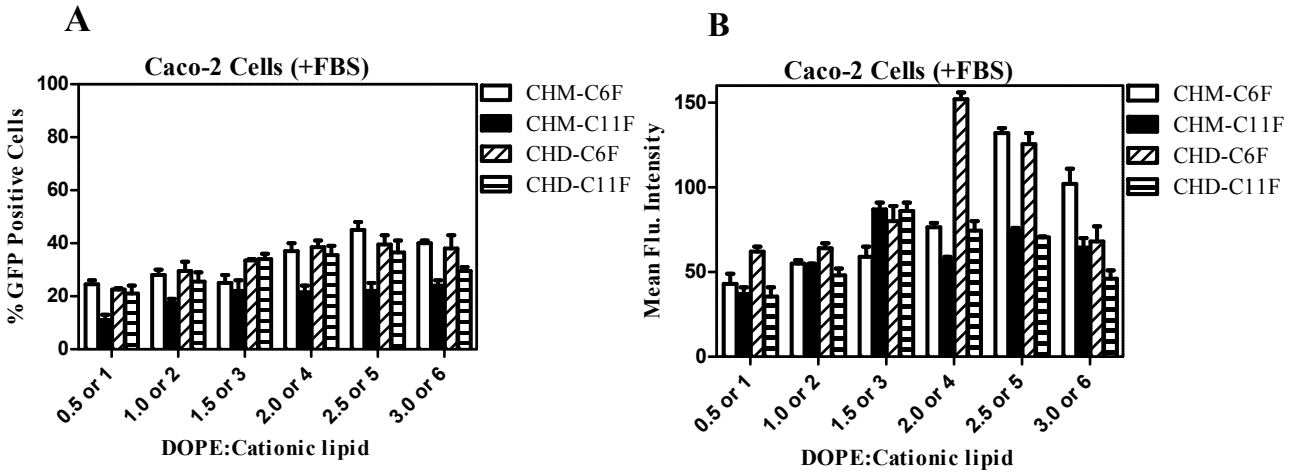

Fig. S4 Optimization of appropriate DOPE ratios for the transfection studies in Caco-2 cells. GFP expression profile is represented as (A) % GFP positive cells and (B) mean fluorescence intensity (MFI). Transfection studies were performed using *p*DNA (pEGFP-C3, 0.8 μg) in 10% FBS containing cell culture medium and GFP expression was analyzed 48 h post transfection. Ratios in x-axis: 0.5, 1, 1.5, 2 and 2.5 for monomeric lipids; 1, 2, 3, 4, 5 and 6 for gemini lipids.

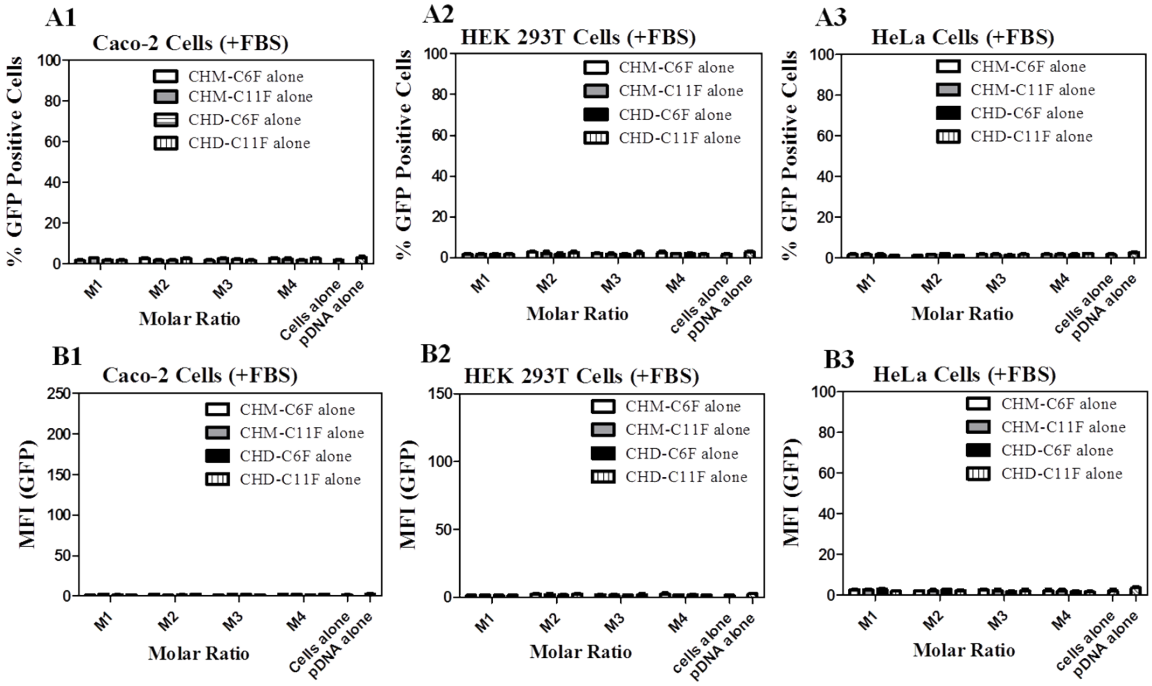

Fig. S5 The GFP expression analysis in terms of % GFP positive cells (A) and MFI (B) in Caco-2 (A1, B1), HEK 293T (A2, B2) and HeLa cells (A3, B3) for various control treatments, *i.e.*, cell alone, cells treated with only co-liposomes (at various concentrations used in different molar ratios and cells treated with pDNA alone (0.8 μg).

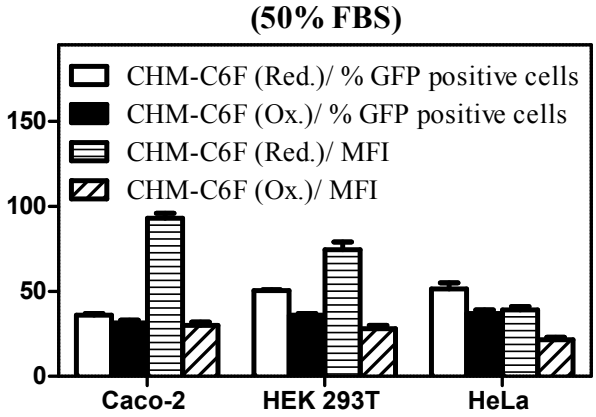

Fig. S6 Transfection efficiency of CHM-C6F (Red. and Ox.) in 50% serum condition in Caco-2, HEK 293T and HeLa cells.

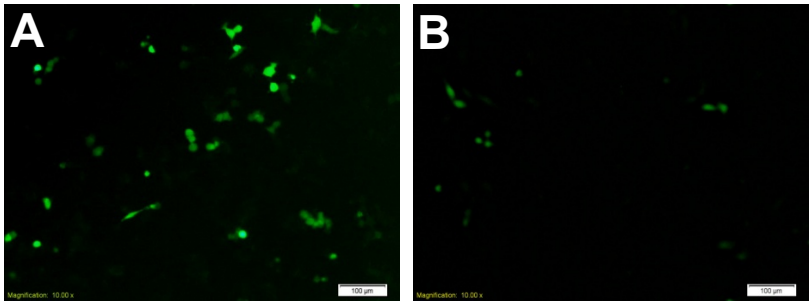

Fig. S7 GFP expression analysis for CHM-C6F co-liposome transfection in reduced (A) and oxidized (B) state of ferrocece in Caco-2 cells in 50% serum condition. GFP expression was analyzed 48 h post transfection.

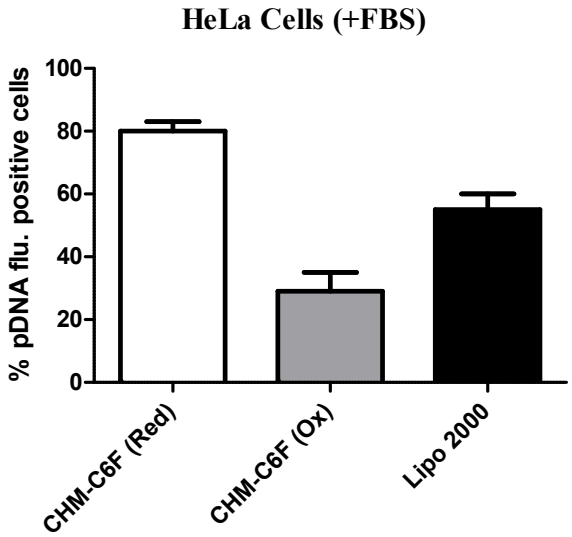

Fig. S8 Flow cytometry analysis for labelled *p*DNA transfection (6 h incubation) mediated by CHM-C6F (Red. and Ox.) in HeLa cells in the presence of 10% serum.

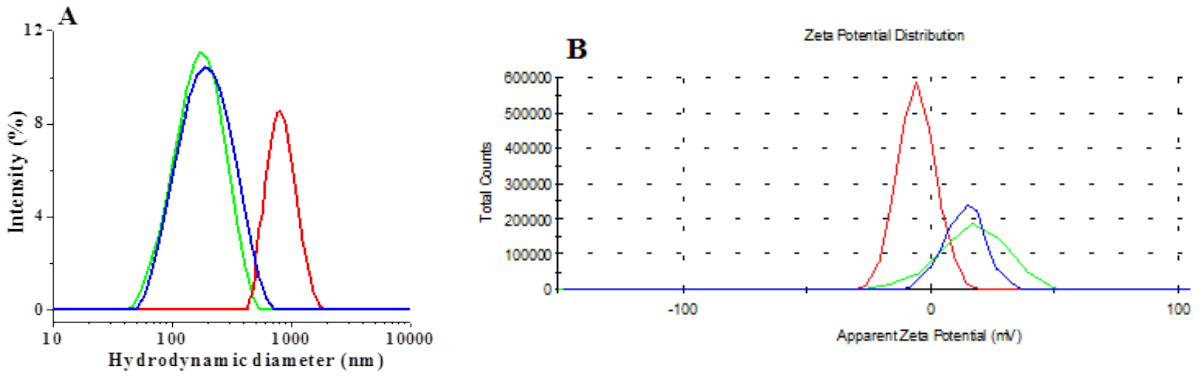

Fig. S9 Hydrodynamic diameters (A) and zeta potentials (B) of reduced lipoplexes (green), oxidized lipoplexes (red) and the oxidized lipoplexes that were treated with ascorbic acid (blue). The co-liposomes of CHM-C6F were used for lipoplex preparation at molar ratio of 2.