Electronic Supplementary Information (ESI)

Novel α-tocopherol–ferrocene conjugates for the specific delivery of transgenes in liver cancer cells under high serum conditions

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Synthesis

Tert-butyl (2-bromoethyl)carbamate (4) and tert-butyl (2-(methylamino)ethyl)carbamate (5): The compounds **4** and **5** were synthesized using 2-bromoethylamine hydrobromide by following a reported procedure.[1]

Di-tert-butyl [azanediyl bis(ethane-2,1-diyl)] dicarbamate (6): Compound **6** was synthesized from diethylene triamine by following a literature procedure.[2]

2-Tocopheryloxyethyl bromide (7): Precursor compound **7** was synthesized from a reported procedure.[3] In brief, 2-tocopheryloxyethanol was synthesized from α-tocopherol by a reaction with ethyl bromoacetate, sodium hydride in DMF followed by reductionof the corresponding ester group by lithium aluminum hydride. The next two-step was tosylation of 2-tocopheryloxyethanol using *p*-TsCl and subsequent bromination with LiBr in acetone to give the titled compound **7**.

Reaction procedure for 8a: Synthesized according to the reported procedure.[4]

Reaction procedure for 8b: Synthesis of 8b followed the same procedure of 8a except that di-tert-butyl (azanediylbis(ethane-2,1-diyl))dicarbamate was used in place of *N-tert* butoxycarbonyl-2-methylamino-ethylamine. The isolated yield was 0.7g, 50%.

Charecterization of 8b: ^{1}H NMR (400 MHz, $CDCl_3$) δ 0.87-0.9 (*m*, 12H, -CH-CH_3, phytyl chain), 1.12-1.56 (*m*, 33H), 1.81-1.83 (*m*, 2H), 2.09 (*s*, 3H, -CH_3), 2.22 (*s*, 3H, -CH_3), 2.26 (*s*, 3H, -CH_3), 2.33 (*s*, 3H), 2.62 (*t*, 2H), 3.69 (*t*, 4H), 4.08 (*m*, 4H), 4.13 (t, 2H), 4.18 (t, 2H); HRMS (ESI) m/z calcd for $[C_{45}H_{81}N_3O_6+H]^+$: 760.613; found: 760.62.

General reaction procedure for 9a/9b: To a solution of compound **8a/8b** (1.9 mmol) dissolved in dry THF, methyl iodide (1 mL) and potassium carbonate (2.9 mmol) were added. The reaction mixture was refluxed for 8h at 60 °C. After the reaction, the solvent was filtered, and evaporation of the filtrate gave the solid crude product. The crude product was purified by flash column chromatography over neutral alumina with chloroform and methanol (v/v 100:4) as eluent. The isolated yield was 0.9g, 90% for **9a** and 0.74g, 90% for **9b**.

Characterization of 9a: Well-matched with an earlier report.[4]

Characterization of 9b : ^{1}H NMR (400 MHz, $CDCl_3$) δ 0.87-0.9 (*m*, 12H, -CH-CH_3, phytyl chain), 1.12-1.56 (*m*, 33H), 1.81-1.83 (*m*, 2H), 2.09 (*s*, 3H, -CH_3), 2.22 (*s*, 3H, -CH_3), 2.26 (*s*, 3H, -CH_3), 2.62 (*t*, 2H), 3.33 (*s*, 3H), 3.69 (*t*, 4H), 4.08 (*m*, 4H), 4.13 (t, 2H), 4.18 (t, 2H); HRMS (ESI) m/z calcd for $[C_{46}H_{84}N_3O_6]^+$: 774.635; found: 774.631.

General reaction procedure of 10a/10b: Compound **9a/9b** was dissolved in 4 N HCl in dioxane (12 mL) at 0°C and stirred at room temperature for 1.5 h. After completing the reaction, the solvent was removed in a vacuum, and the residue was washed with diethyl ether (20 mL). The precipitate was used for the next step without further purification. The yield for them was 0.61g, 85% for 10a and 0.47g, 80% for 10b.

Charecterization of 10a: Well-matched with earlier report.[4]

Charecterization of 10b: ^{1}H NMR (400 MHz, CD_3OD)δ 0.87-0.9 (*m*, 12H, -CH-CH_3, phytyl chain), 1.12-1.56 (*m*, 24H), 1.81-1.83 (*m*, 2H), 2.09 (*s*, 3H, -CH_3), 2.22 (*s*, 3H, -CH_3), 2.26 (*s*, 3H, -CH_3), 2.62 (*t*, 2H), 3.33 (*s*, 6H), 3.69 (*t*, 4H), 4.08 (*m*, 4H), 4.13 (t, 2H), 4.18 (t, 2H); HRMS (ESI) m/z calcd for $[C_{36}H_{68}N_3O_2]^+$: 574.531; found: 574.531.

Synthesis of ferrocene dialdehyde (11): It was synthesized from ferrocene following a reported literature procedure.[5]

Synthesis of 12: This was synthesized by following a literature procedure from its synthesized precursor **11**.[6]

Charecterization of 12: ^{1}H NMR (400 MHz, d_6-DMSO) δ 4.37(*s*, 4H), 4.57 (*s*, 4H), 5.94-5.98 (*d*, 2H), 7.24-7.28 (*d*, 2H); HRMS (ESI) m/z calcd for $[C_{16}H_{14}FeO_4 + Na]^+$: 349.119; found: 349.119.

Reaction procedure of 13: Compound was synthesized by hydrogenation reaction. Precursor compound **12** was taken in dry ethyl acetate solution followed by adding 10% Pd/C under N_2 atmosphere. After that, the reaction was continued for 6 hours under a hydrogen atmosphere to achieve the dehydrogenated product **13**. Yield 70%.

Charecterization of 13: ^{1}H NMR (400 MHz, d_6-DMSO) δ 3.96(*s*, 4H), 3.99 (*s*, 4H), 2.37-2.41 (*m*, 4H), 2.47-2.49 (*m*, 4H); HRMS (ESI) m/z calcd for $[C_{16}H_{18}FeO_4 + H]^+$: 331.169; found: 331.169.

Synthesis of 14 & 15: It was synthesized by following a literature procedure from commercially available ferrocenaldehyde.[5]

General reaction procedure for the syntheses of lipids, monomer TMF and TDF: Both compounds were synthesized by following the amide coupling reaction. Briefly, reactant **10a**/**10b** (amine part) was taken in dry THF under the N_2 atmosphere, followed by addition of 1.5/2.5 equivalent of Et_3N. The mixture was kept on stirring on an ice-bath for 30 minutes. In a separate round bottom flask compound 15 (acid part) was taken in 1.5 equivalent of **10a** (for TMF) or 3.5 equivalent of **10b** (for TDF) in dry DMF under N_2 atmosphere. This was followed by addition of hydroxybenzotriazole (HOBt) and 1-ethyl-3-(3-dimethylamino-propyl)carbodiimide (EDC), to this solution was also kept on stirring conditions on an ice bath for 30 min. After 30 min of stirring, the solution of 10a/10b in THF was transferred to the reactant 15 and the combined reaction mixture was kept on stirring under nitrogen at room temperature for 24 h. After keeping the reaction for one day, it was quenched with 1N HCl followed by extraction of the compound using ethyl acetate was performed. The organic layer was further washed with brine and distilled water. The combined organic layer was passed through anhydrous sodium sulfate, and the solvent was evaporated using a rotatory evaporater. The crude product was further purified over an alumina column using eluting solvent mixture of chloroform and methanol (v/v 100:6). yield was 0.2g, ~70% for TMF and 0.25g, ~65 % for TDF.

Characterization of TMF: FT-IR (Neat, cm^{-1}) 2929, 2884, 2796, 1711, 1505, 1458, 1369, 1253, 1169, 1089, 1058, 921. ^{1}H NMR (400 MHz, $CDCl_3$) δ 0.84-0.88 (*m*, 12H, -CH-CH_3, phytyl chain), 1.08-1.58 (*m*, 24H), 1.78-1.8 (*m*, 2H), 2.04 (*m*, 2H, -CH_2), 2.09 (*s*, 3H, -CH_3), 2.11 (*s*, 3H, -CH_3), 2.15 (*s*, 3H, -CH_3) 2.5 (*t*, 2H), 2.57 (*t*, 2H), 2.69 (*t*, 2H), 3.45 (*s*, 6H), 3.83 (*m*, 2H), 3.91-3.92 (*m*, 2H), 3.99 (*t*, 2H), 4.03 (*s*,4H), 4.1 (*s*, 5H); ^{13}C NMR (100 MHz, $CDCl_3$) *δ*. 11.82, 12.39, 13.24, 19.65, 19.72, 20.65, 21, 22.6, 22.69, 23.48, 23.71, 24.4, 24.75,25.29,27.93, 32.73, 34.07, 37.4, 39.33, 40.03, 52.72, 64.52, 67.2, 68.26, 68.62,87.68, 117.91, 123.42, 124.98, 126.79, 147.34, 173.89; HRMS (ESI) m/z calcd for $[C_{48}H_{77}FeN_2O_3]^+$:

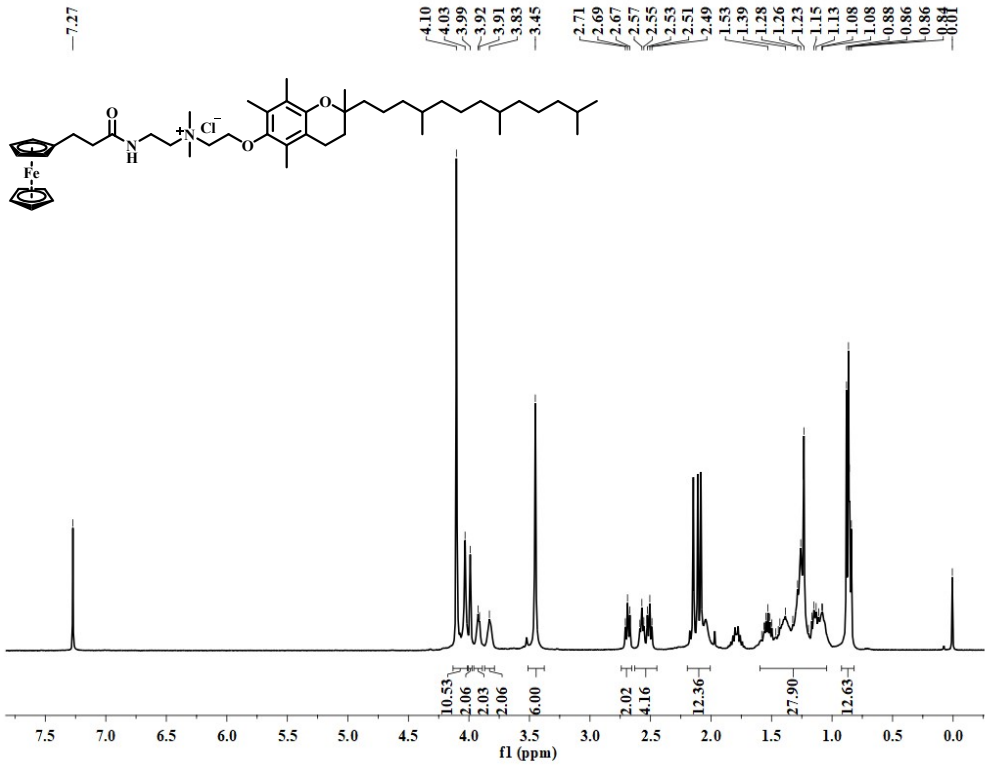
785.5278; found: 785.5485, Anal. Calcd for $C_{48}H_{77}FeN_2O_3Cl_2.2.7H_2O$: C 66.26, H 9.55, N 3.22. Found: C 66.23, H 9.52, N 3.26.

Characterization of TDF: FT-IR (Neat, cm^{-1}) 3379, 2926, 2861, 1702, 1508, 1459, 1370, 1252, 1169, 1090, 1019, 954. ^1H NMR (400 MHz, CD_3OD) δ 0.85-0.89 (*m*, 12H, -CH-CH_3, phytyl chain), 1.09-1.54 (*m*, 24H), 1.55-1.65 (*m*, 2H), 2.1 (*s*, 3H, -CH_3), 2.11 (*s*, 3H, -CH_3), 2.15 (*s*, 3H, -CH_3), 2.51 (*t*, 4H), 2.59 (*t*, 2H), 2.67 (*t*, 4H), 3.5 (*s*, 6H), 3.7-3.9 (*m*, 10H), 4 (*s*, 6H), 4.11 (*s*, 12H); ^{13}C NMR (100 MHz, $CDCl_3$) δ. 11.85, 12.43, 13.28, 19.67,19.74, 20.67, 21.02, 22.61, 22.7, 23.72, 24.41, 24.75,24.77, 25.31, 27.94, 32.73, 34.2, 37.35, 37.4, 37.5, 39.3, 50.76, 61.91, 62.91, 65.55, 67.25, 68.2, 68.63, 75.06, 87.57, 117.91, 123.46, 124.99, 126.8, 147.25, 148.3, 173.83; HRMS (ESI) m/z calculated for $[C_{62}H_{92}Fe_2N_3O_4]^+$: 1054.578; found: 1054.619; Anal. Calcd for $C_{62}H_{92}Fe_2N_3O_4Cl_2.3.1H_2O$: C 64.96, H 8.63, N 3.67. Found: C 64.91, H 8.58, N 3.65.

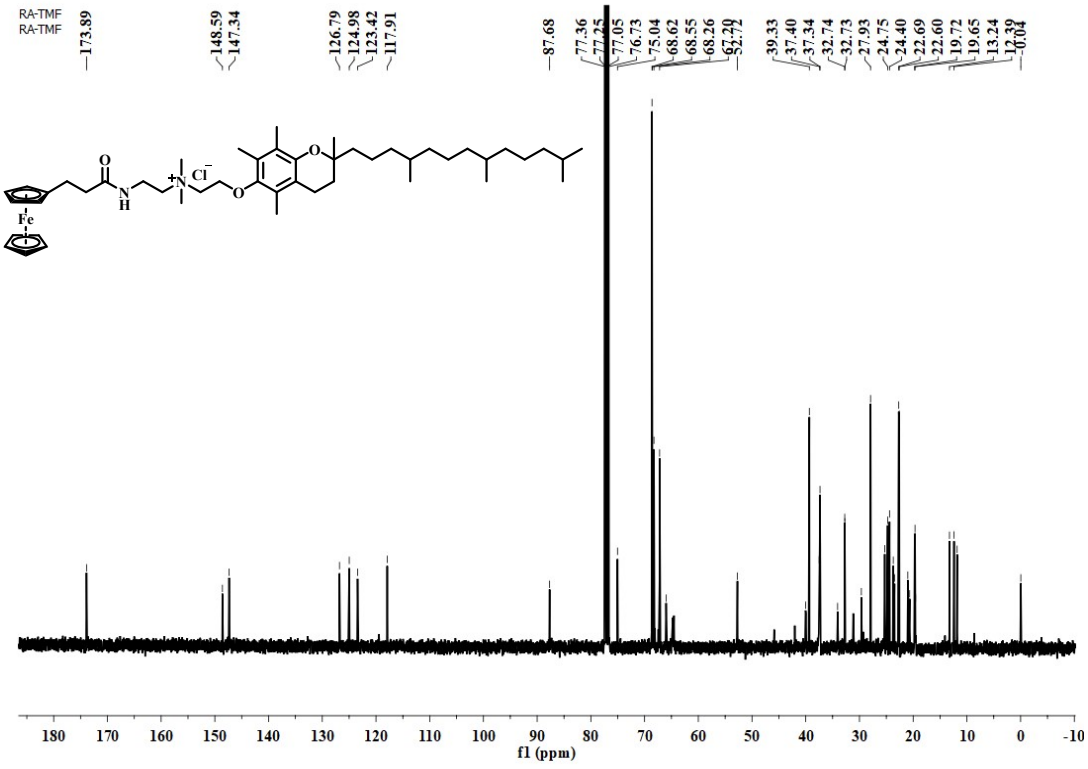
Reaction procedure of Gemini lipid T2F: The reaction procedure was the same as discussed above. The only difference was that the acid part was compound 13, and the amine part was 10a. The compound was purified by alumina column by eluting chloroform-methanol mixture (v/v=10:1). The yield was 0.1g, 60%.

Characterization of T2F: FT-IR (Neat, cm^{-1}) 3407, 2925, 2861, 1460, 1374, 1235, 1163, 1092, 1021. ^1H NMR (400 MHz, $CDCl_3$) δ 0.8-0.84 (*m*, 24H, -CH-CH_3, phytyl chain), 1.04-1.52 (*m*, 54H), 1.73-1.84 (*m*, 4H), 2.03 (*s*, 6H, -CH_3), 2.07 (*s*, 6H, -CH_3), 2.11 (*s*, 6H, -CH_3), 2.52 (*m*, 4H), 2.57 (*m*, 2H), 2.65 (*m*, 2H), 3.54 (*s*, 12H), 3.81 (*br*, 2H), 3.93-3.98 (*d*, 8H), 4.04-4.09 (m,8H), 4.28-4.36 (m, 2H); ^{13}C NMR (100 MHz, $CDCl_3$) δ. 11.7, 12.7, 13.4, 19.5, 19.6, 19.7, 20.6, 21, 22.6, 22.7, 23.4, 24.4, 24.7, 31.2, 31.3, 32.7, 34.2, 37.2, 37.3, 37.4, 37.5, 37.6, 39.3, 40.2, 40.3, 52.6, 62.7, 65.2, 66.3, 74.8, 117.7, 123, 125.2, 127, 147.6, 148.3; HRMS (ESI) m/z calcd for $[C_{86}H_{144}FeN_4O_6]^{2+}$m/z= 692.5212; found: 692.5055. Anal. Calcd for $C_{86}H_{144}FeN_4O_6Cl_2.6.7H_2O$: C 65.48, H 10.05, N 3.54. Found: C 65.47, H 10.09, N 3.57.

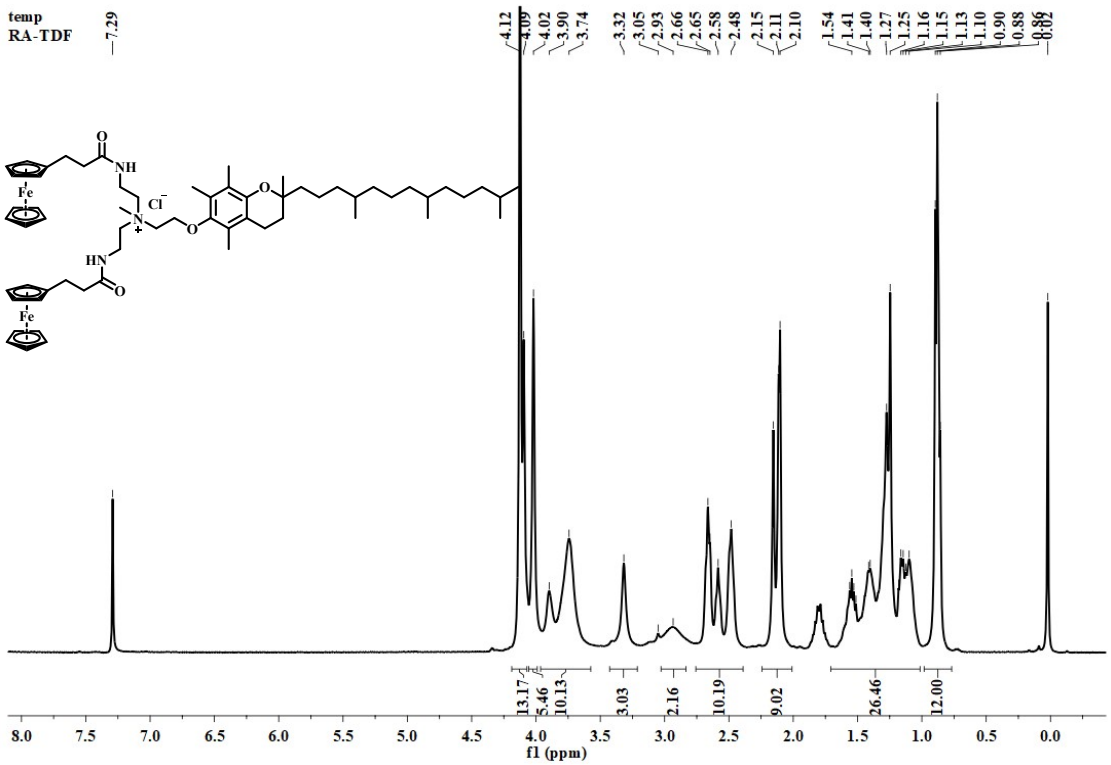
^{1}H NMR spectra of TMF (2)



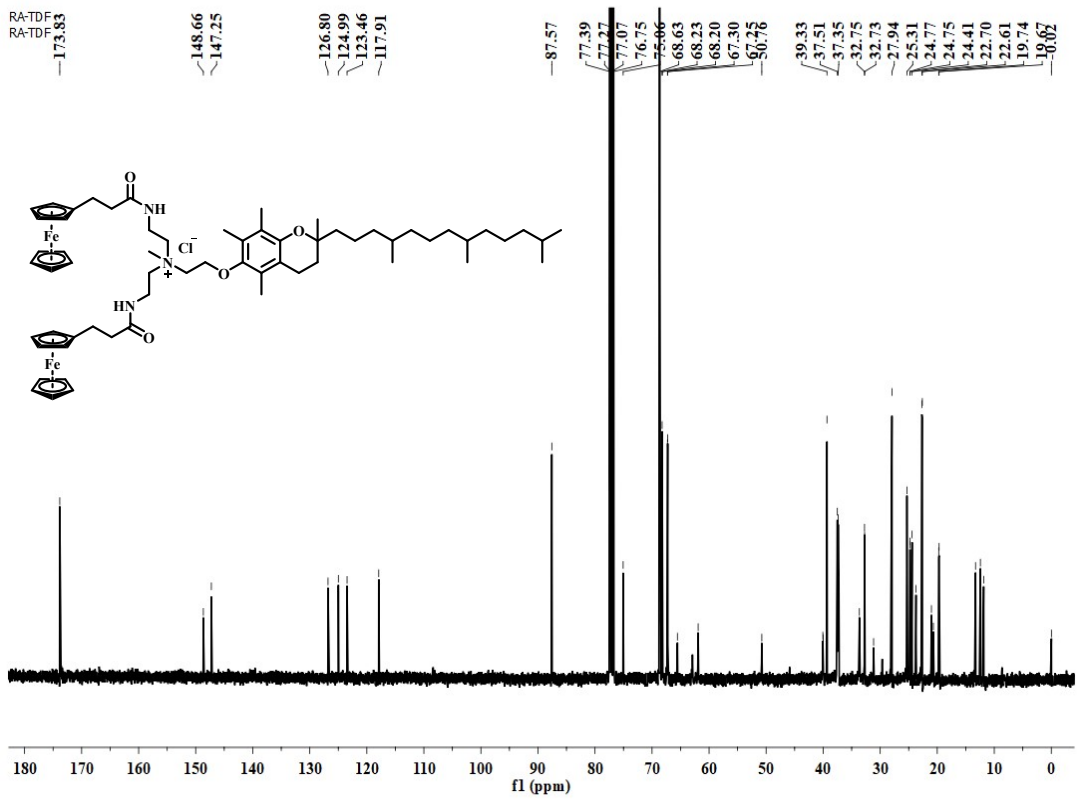
^{13}C NMR spectra of TMF (2)



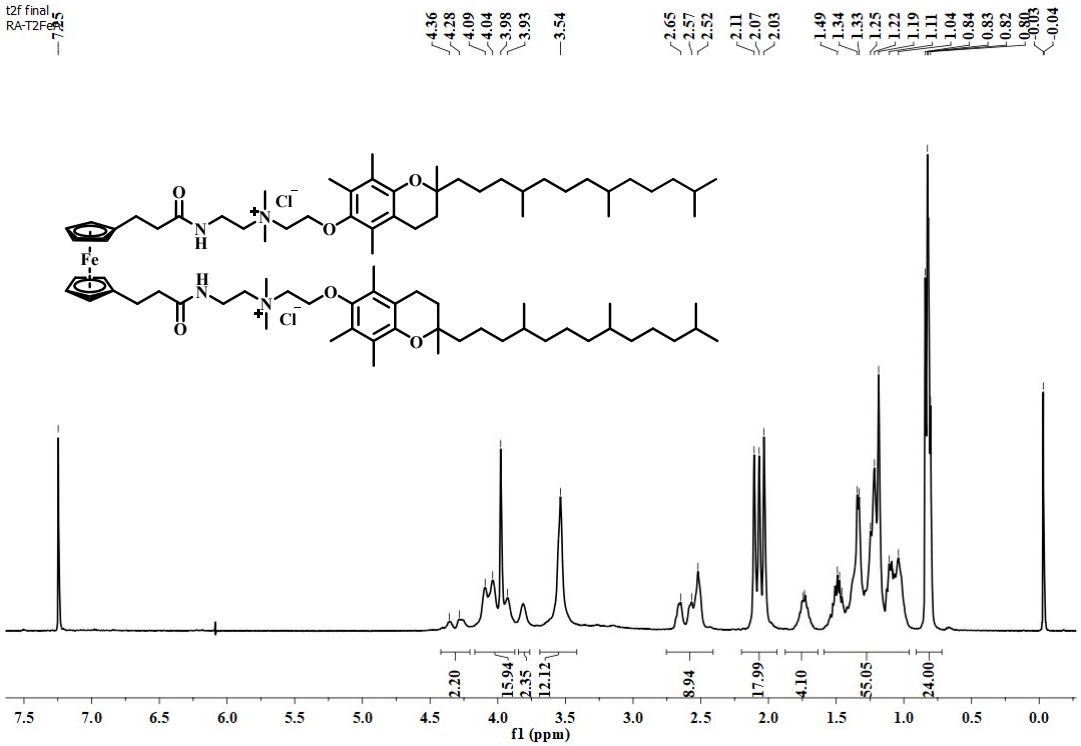
^{1}H NMR spectra of TDF (3)



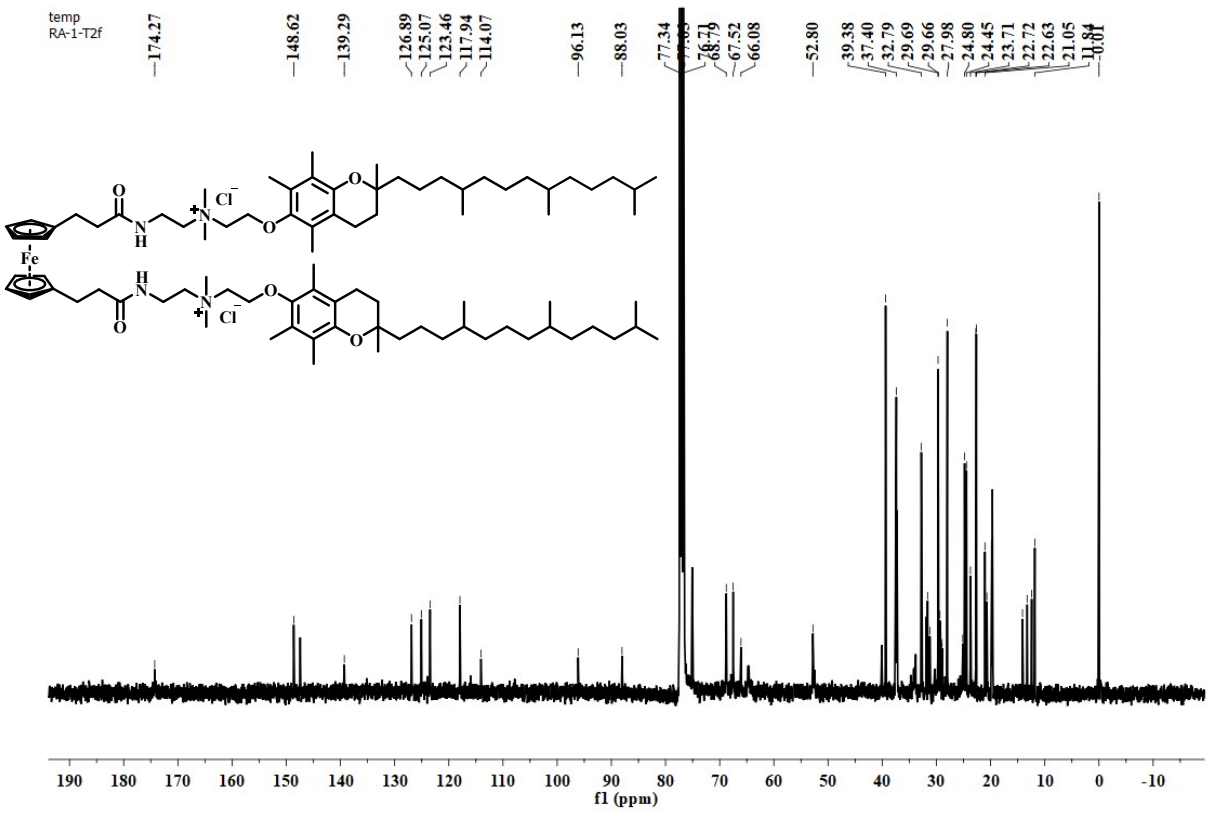
^{13}C NMR spectra of TDF (3)



^{1}H NMR spectra of T2F (1)



^{13}C NMR spectra of T2F (1)



Oxidation of ferrocene-tocopherol conjugates: Oxidation of ferrocene was done with the help of $FeCl_3$. Briefly, each reduced monomer or gemini lipid was dissolved in methanol-chloroform (v/v 1:1, 2 mL) mixture then a fixed amount of methanolic solution of $FeCl_3$ was added to this. Conversion of the color yellow to green was observed. After that solvent was evaporated and dissolved again in chloroform. By centrifugation, excess $FeCl_3$ was removed. Oxidation of ferrocene moiety of all molecules was confirmed by UV-vis spectroscopy in chloroform.

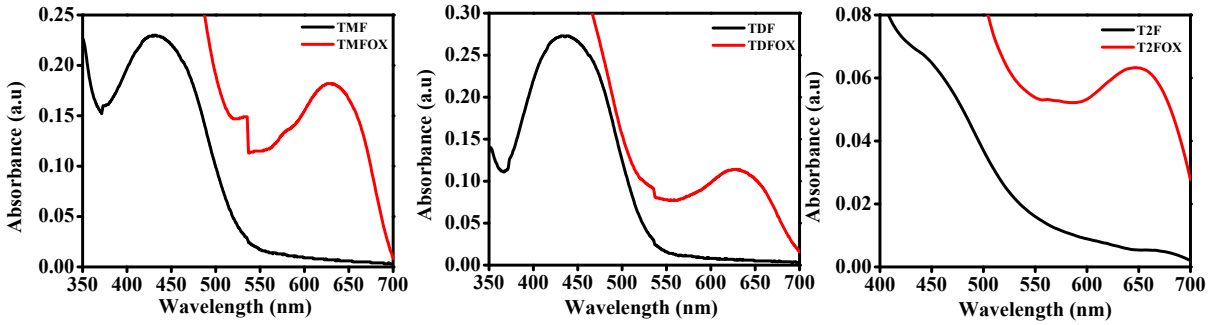


Fig. S1. UV-Vis spectra of redox-active molecules (TMF, TDF, T2F) in reduced (black) and oxidized (red) states (in chloroform).

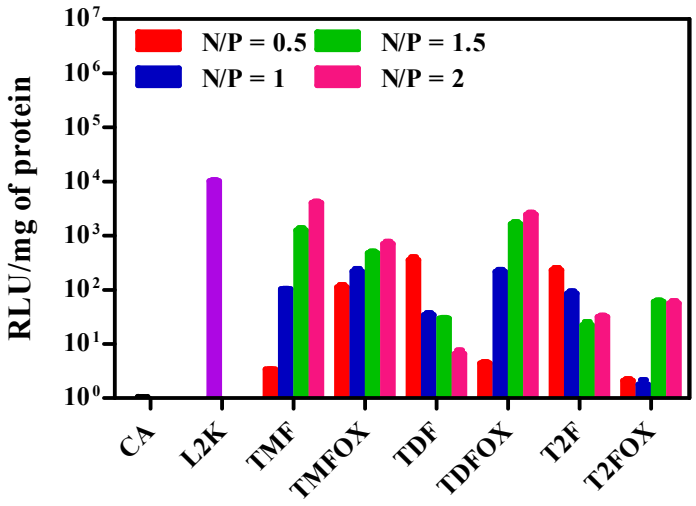


Fig. S2. Expression of luciferase protein with lipoplexes of different tocopherol-ferrocene conjugates (lipids) at N/P ratios 0.5 - 2 in HepG2 cells.

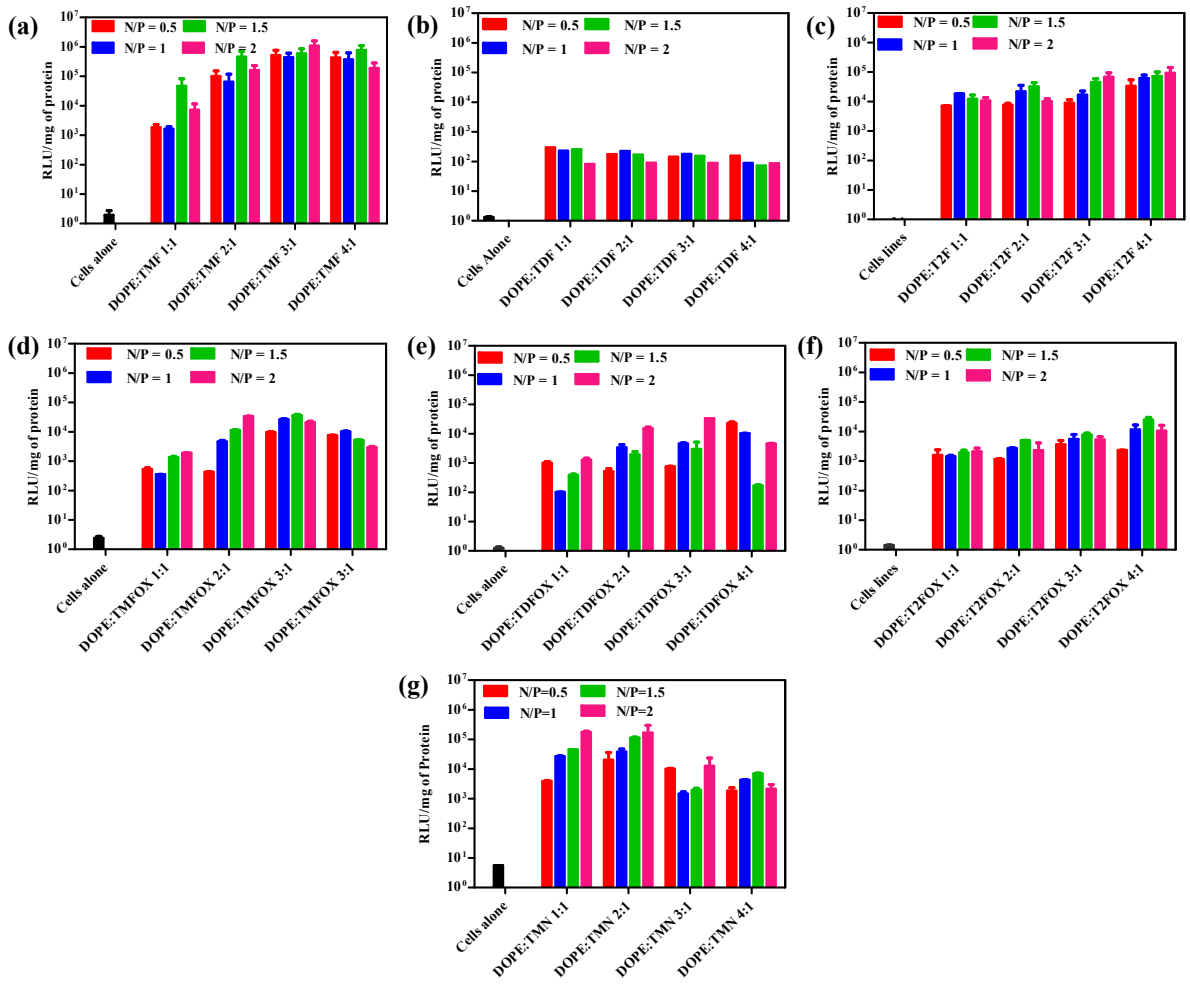


Fig. S3. a-g**.** Optimization of DOPE content of different DOPE-monomer/gemini lipid co-liposomes in HepG2 cell for luciferase protein expression. Data for DOPE molar ratios 1:1 to 4:1 at N/P ratios 0.5, 1, 1.5, and 2 are shown here.

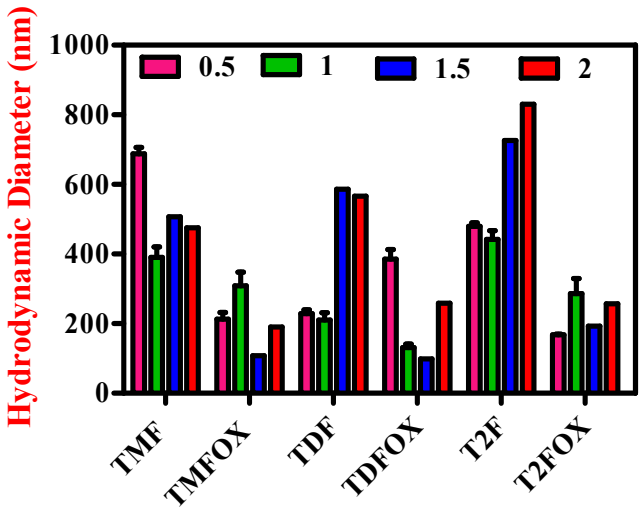


Fig. S4. Hydrodynamic diameters of all optimized lipoplexes at N/P ratio 0.5-2.

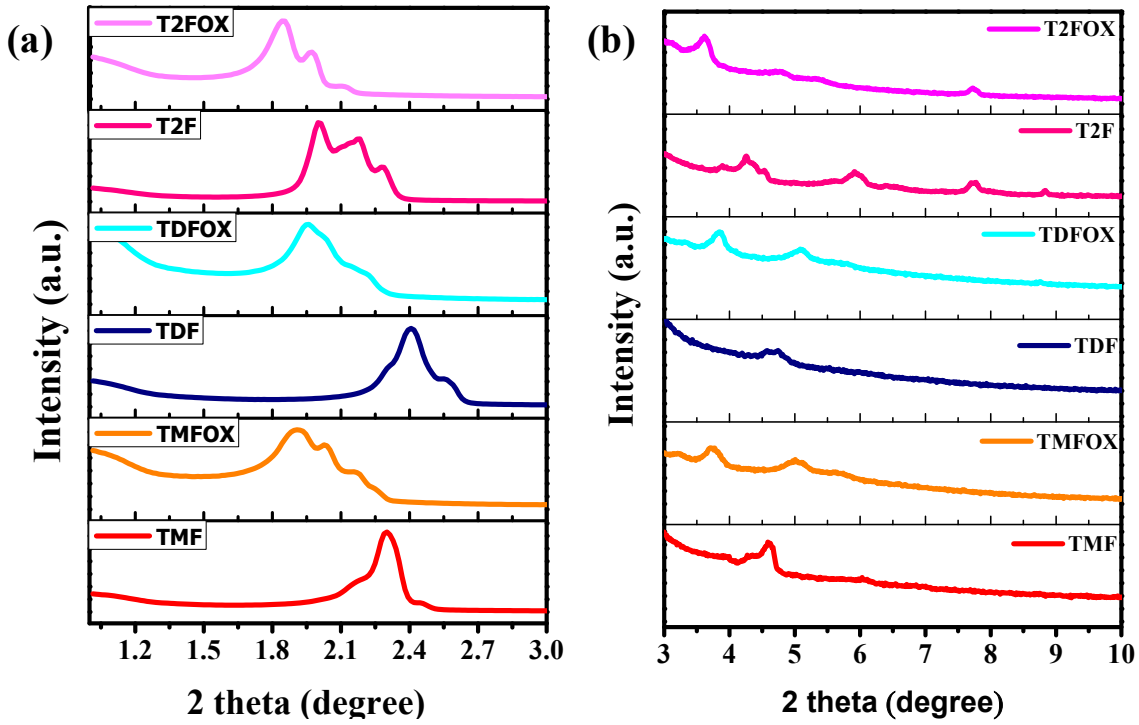


Fig. S5: XRD data of optimized co-liposomes (a) First order diffraction peak (b) Higher-order diffraction peaks (Optimized co-liposomes DOPE-TMF/TMFOX=3:1, DOPE-TDF/TDFOX=3:1, DOPE-T2F/T2FOX=4:1).

Table S1: The tabular X-RD data of first-order diffraction peak (2θ) and the corresponding their bilayer width of all presentation of the optimized co-liposomes.

Compounds	2θ (degree)	d_{100}(Å)
TMF	2.3	38.4
TMFOX	1.9	46.49
TDF	2.4	36.81
TDFOX	1.96	45.07
T2F	2	44.17
T2FOX	1.84	48.01

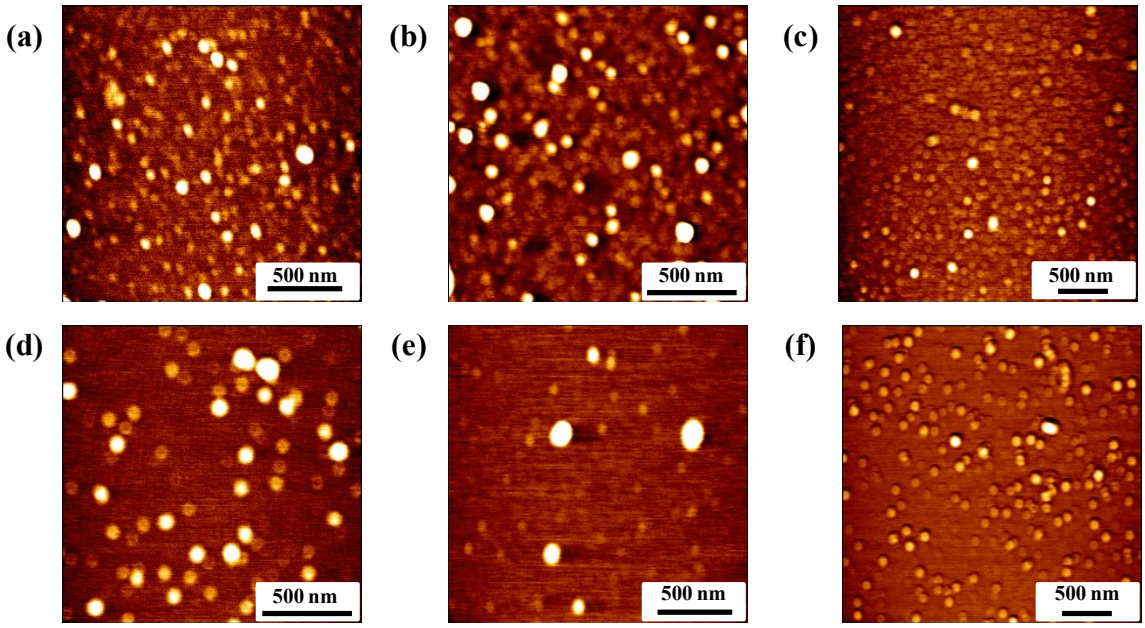


Fig. S6. Representative AFM images of optimized co-liposomes (a) TMF (b) TDF (c) T2F (d) TMFOX (e) TDFOX (f) T2FOX (Optimized co-liposomes DOPE-TMF/TMFOX=3:1, DOPE-TDF/TDFOX=3:1, DOPE-T2F/T2FOX= 4:1).

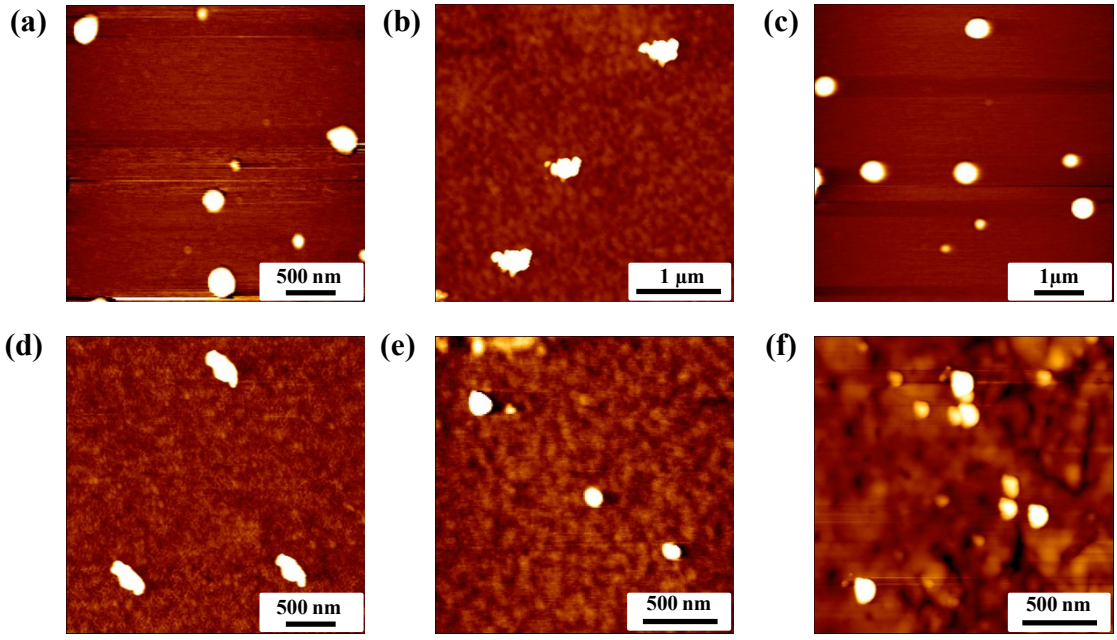


Fig. S7. Representative AFM images of lipoplex of optimized co-liposomes at N/P ratio 2 (a) TMF (b) TDF (c) T2F (d) TMFOX (e) TDFOX (f) T2FOX (Optimized co-liposomes DOPE-TMF/TMFOX=3:1, DOPE-TDF/TDFOX=3:1, DOPE-T2F/T2FOX=4:1).

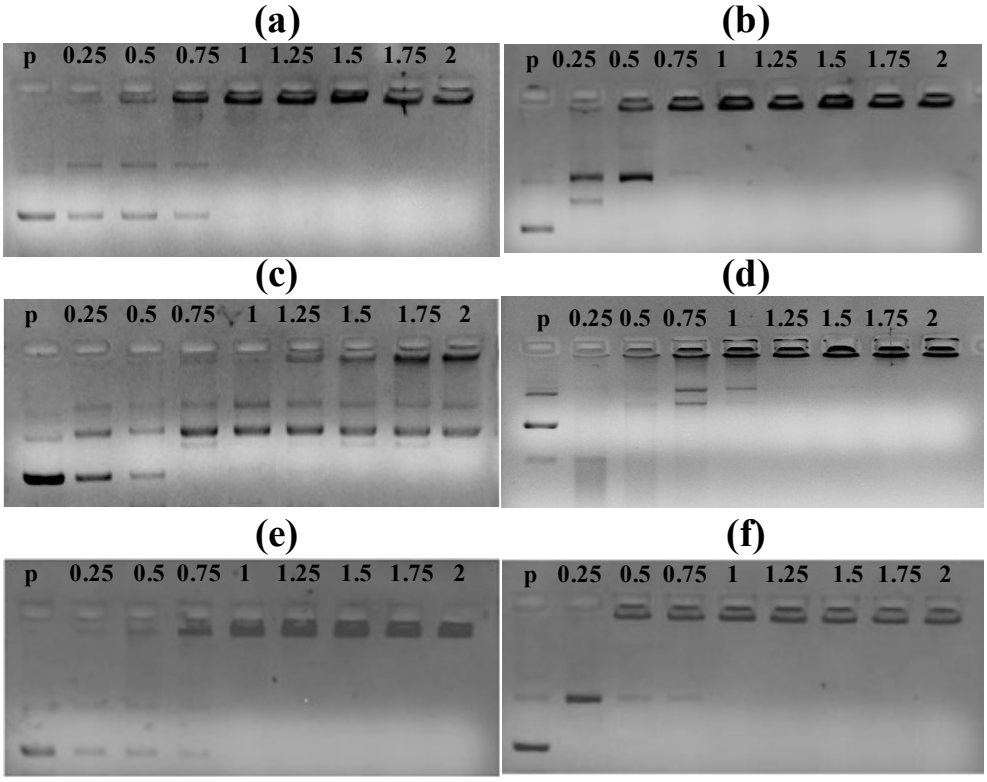


Fig. S8. Gel electrophoresis images for the lipoplexes of co-liposome (a) TMF, (B) TMFOX, (c) TDF, (d) TDFOX, (e) T2F, and (f) T2FOX (DOPE-TMF/TMFOX and TDF/TDFOX = 3:1, DOPE-T2F/T2FOX = 4:1). The co-liposome was complexed with *p*DNA (0.2 μg) and run on 1% agarose gel for 30 min. (p = *p*DNA).

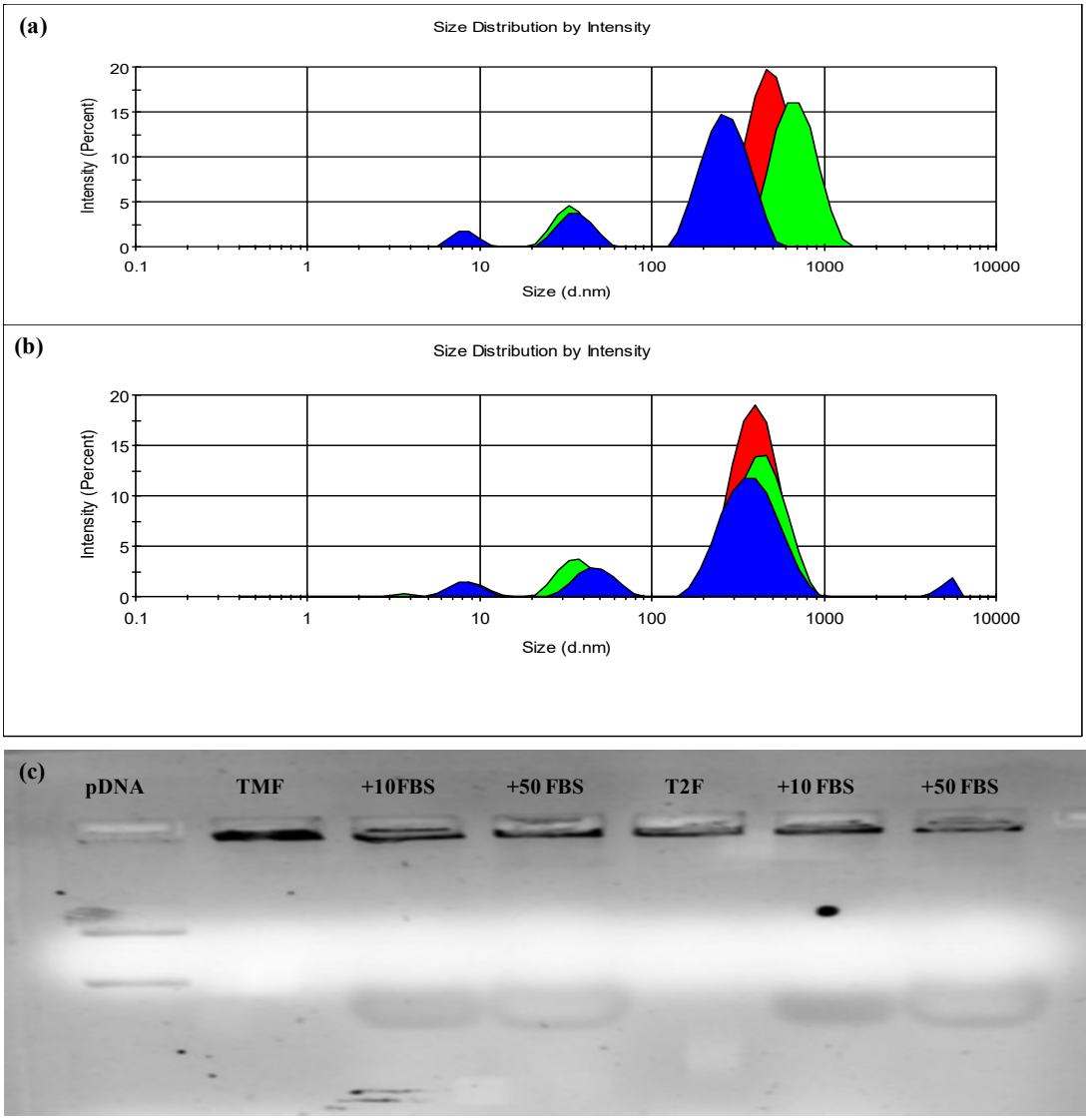


Fig. S9. Changes in the hydrodynamic diameter of the optimized lipoplex formulations after addition of FBS at N/P ratio of 2 (a) TMF and (b) T2F. (Red Area = lipoplex alone, green area = lipoplex + 10% FBS, Blue area = lipoplex + 50% FBS). (c) Gel retardation assay of TMF and T2F after addition of 10% and 50% FBS.

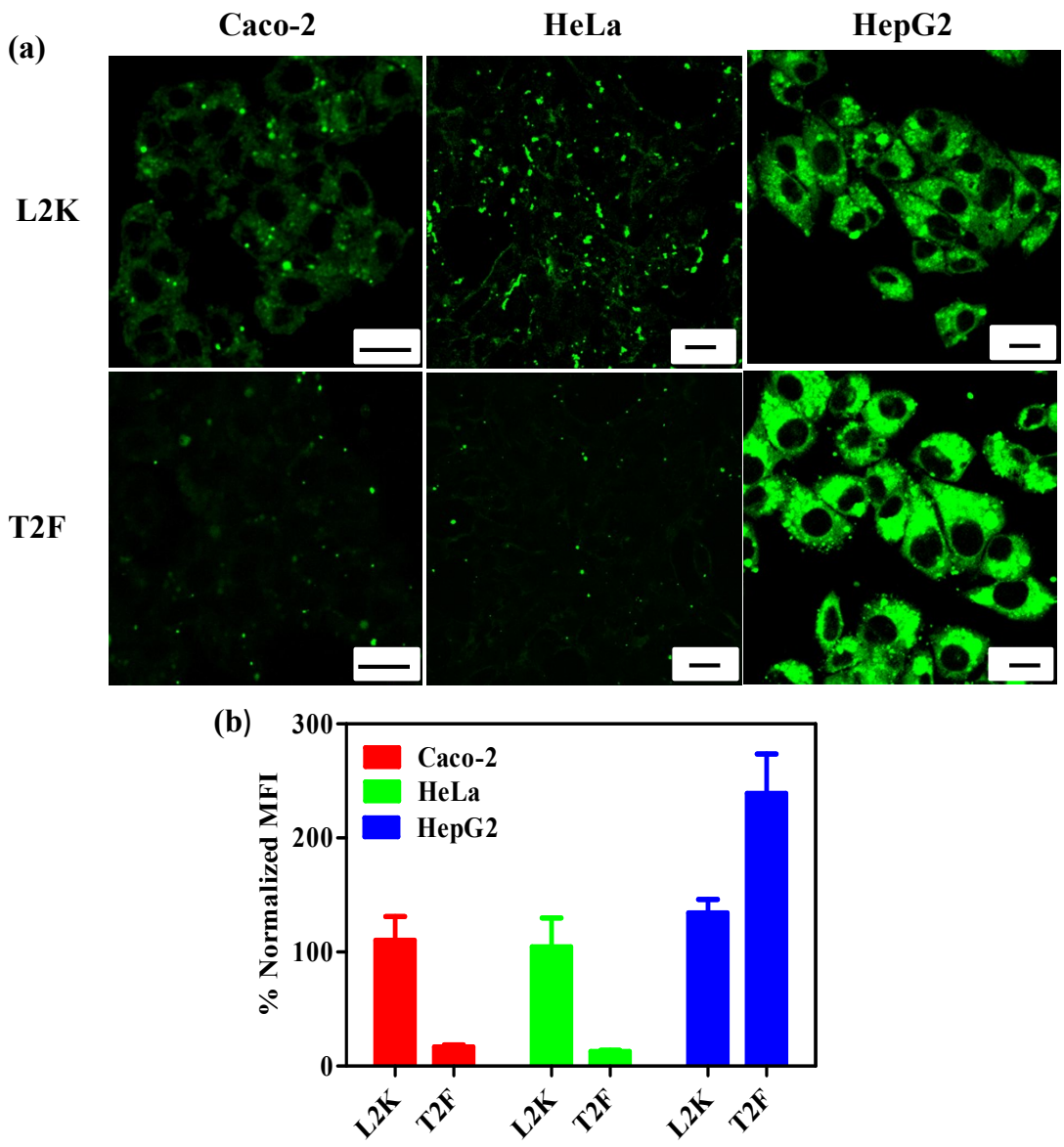


Fig. S10. (a) Confocal images of fluorescein tagged plasmid after internalization and (b) their comparison of the amount of internalized pDNA (in term of fluorescence intersity) with L2K and DOPE-T2F (4:1) formulations in different cells(Scale bar 20µm).

References:

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