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Supporting Information

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17β-Estradiol Associated Stealth-Liposomal Delivery of Anticancer Gene to Breast Cancer Cell**

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EXPERIMENTAL PROCEDURES

Chemicals and General Procedures:

DSPE-PEG-maleimide was purchased from Shearwater Polymers (Huntsville, AL) and DPPE-PEG-NH2 ($C_{128}H_{258}O_{54}N_3P$, catalogue number 880168, Molecular weight 2739.37) was purchased from Avanti Polar Lipids (Birmingham, AL). Cholesterol, trypsin, EDTA, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), dimethylsulfoxide (DMSO), Estrone (ES) were purchased from Sigma Chemical Co. (St. Louis, MO). All the chemicals and organic solvents required for synthesis were purchased from either Aldrich (Milwaukee, WI, USA) or S. D. Fine Chem (Mumbai, India). They were used without further purification. CMV-sport b-gal plasmid and CMV-p53 plasmid were obtained as kind gifts from Dr. N. Madhusudhana Rao (Center for Cellular and Molecular Biology, Hyderabad, India) and Dr. Robert J. Debbs (University of Southern California, California, USA) respectively. Tamoxifen molecule was obtained and purified from drug, Tamoxifen citrate I.P. (Medibios Laboratories Pvt Ltd., Mumbai, India & Dabur India Ltd. Ghaziabad, India). Briefly, Tamoxifen citrate tablets, (10 X 10mg by Tamoxifen weight) were crushed and taken in 10 ml of Acetone and filtered. Acetone filtrate was evaporated to get the free drug as residue. The residue was subjected to crystallization in methanol/ether

(2:13 v/v) solvent mixture at -20^0 C. The purity and authenticity of crystallized compound (white powder, 58 mg) was characterized by TLC, melting point analysis (M.P. 141^0 C) and by NMR spectrum. All the ^{1}H NMR spectra were recorded on a Bruker FT 300 MHz and Varian FT 200 MHz instrument. Mass spectral analysis was done either in FAB Mass (Autospec, Manchester, UK), MALDI TOF mass and ESI Mass.

Cell Culture:

MCF-7, CHO, Hela, L-27, MDA-MB 231 and T47D cells were purchased from National Center for Cell Sciences (Pune, India) and were mycoplasma free. Cells were cultured in DMEM medium [for MDA-MB 231 cells, L-15 Leibovitz medium] containing 10% fetal bovine serum (Sigma Chemical Co., St. Louis, MO) and 1% penicillin-streptomycin-Kanamycin at 37^0C in a humidified atmosphere of 5% CO_2 in air. Cultures of 85-90% confluency were used for all of the experiments. The cells were trypsinized, counted, subcultured in 96-well plates for transfection and viability studies. The cells were allowed to adhere overnight before they were used for experiments.

Syntheses of Ligands: The synthetic procedure for preparing the lipid DPPE-PEG-ES is depicted schematically in Figure S1. Detailed experimental procedures are delineated below.

Step A: Synthesis of 3-tert butyldimethylsilyloxy Estrone: To a solution of estrone (1g, 3.69 mmoles) in dry DCM & dry DMF (9:1 v/v), 1.18g (11.09 mmoles) of 2,6 Lutidine and 0.93ml (4.06 mmoles) of TBDMS-triflate at 0^0 C was added**.** The reaction mixture was stirred at room temperature for 30 min. The reaction mixture was taken in 20 ml of dichloromethane, washed with saturated $NaHCO_3$ solution (1 x 20 ml), water (2 x 20 ml) and brine (1 x 20ml) The non-aqueous solvent was finally dried with anhydrous Na_2SO_4 and dried in vacuum. Column chromatographic purification (using 60-120 mesh silica gel and 2% ethyl acetate in hexane as eluting solvent mixture) of the dried residue afforded **I** as a white solid (1.01 g, 71% yield, R_f 0.4 in 10% ethyl acetate in hexane).

^{1}H NMR (300 MHz, $CDCl_3$) **:** δ =0.19 [s, 6H, $Si(C\underline{H}_3)_2$], 0.9 [s, 3H, 18-C<u>H</u>3], 1.0 [s,9H, $SiC(C\underline{H}_3)_3$], 1.4-1.7 and 1.9-2.55 [m, 13H, cyclopentane and cyclohexane ring protons], 2.85 [m, 2H, 6-$C\underline{H}_2$], 6.52 [d, J=2.2 Hz ,1H, 4-C<u>H</u>], 6.57 [dd, J1=2.6 Hz and J2=8.4 Hz ,1H, 2-C<u>H</u>], 7.09 [d, J=8.4 Hz,1H, 1-C<u>H</u>],

FABMS (LSIMS): m/z: 384 [M^+] for $C_{24}H_{36}SiO_2$

Step B: Synthesis of 3-tert butyldimethylsilyloxy-17α-propenyl-17β-Estradiol: To a mixture of Mg metal (300mg, 12.57 mmoles) in dry THF (5ml), 1,2-dibromoethane (catalytic amount) and Allyl bromide (1.07ml, 12.57mmoles) was added drop wise at 0^0 C under Nitrogen atmosphere. The reaction mixture was stirred at room temperature for 30

min. To the above mixture **I** (967mg, 2.5mmoles) dissolved in dry THF (10ml) was added at 0^0 C. The reaction mixture was refluxed for 16h. After the completion of the reaction the reaction mixture was quenched by adding saturated solution of Ammonium chloride solution (20ml) at room temperature and subsequently extracted with ethyl acetate (2 x 25ml). The organic layer was washed with water (2x 50 ml), brine (1 x 50ml) and finally dried with anhydrous Na_2SO_4. Column chromatographic purification (using 60-120 mesh silica gel and 4 % ether in hexane as eluting solvent) of the residue afforded **II** as a white solid (890 mg , 83% yield, R_f 0.58 in 20% ether in hexane).

^{1}H NMR (300 MHz, $CDCl_3$) **:** δ =0.19 [s, 6H, Si($C\underline{H}_3$)$_2$], 0.91 [s, 3H, 18-$C\underline{H}_3$], 1.0 [s,9H, SiC($C\underline{H}_3$)$_3$], 1.2-1.7 & 2.05-2.4 [m, 13H, cyclopentane and cyclohexance ring protons], 1.85-2.0 [dm, 2H, -$C\underline{H}_2$-$CH=CH_2$] 2.85 [m, 2H, 6-$C\underline{H}_2$], 5.1-5.32 [dd, 2H, $CH=C\underline{H}_2$], 5.95 [m, 1H, $C\underline{H}=CH_2$], 6.52 [d, 1H, 4-C<u>H</u>], 6.57 [dd, 1H, 2-C<u>H</u>] 7.09 [d, 1H, 1-C<u>H</u>],

FABMS (LSIMS): m/z: 426 [M^+] for $C_{27}H_{42}SiO_2$

Step C: <u>Synthesis of 3-tert-butyldimethylsilyloxy-17α-[3-hydroxypropane]-17β-estradiol</u> : To a stirred DMF (5 ml) solution of 17α-propenyl-β-Estradiol (890 mg, 2.09 mmole), borane in DMS (97% solution, 0.3ml, 4.28 mmoles) was added at 0^0 C and stirred under nitrogen for 6h. A solution of 3N NaOH (6 ml) and H_2O_2 (4.5 ml, 30 % w/v) was added at 0^0 C and the mixture was warmed to room

temperature. The resulting mixture was then quenched by water, and extracted with ethyl acetate (2 X 20 ml). Organic layer was washed with water (2 X 25 ml), brine (1 X 25 ml), finally dried over anhydrous Na_2SO_4 and evaporated under vacuum. The crude compound was purified by column chromatography with hexane/ethyl acetate (80:20) as eluting solvent mixture to give 481mg (51.25%) of Alcohol (**III)**.

^{1}H NMR (300 MHz, $CDCl_3$) **:** δ =0.19 [s, 6H, $Si(C\underline{H}_3)_2$], 0.91 [s, 3H, 18-C<u>H</u>3], 0.99 [s,9H, $SiC(C\underline{H}_3)_3$], 1.2-2.3 [m, 17H, cyclopentane, cyclohexance ring protons & 17α-$C\underline{H}_2C\underline{H}_2$-$CH_2$-OH], 2.80 [m, 2H, 6-$C\underline{H}_2$], 3.6-3.75 [dm, 2H, 17$\alpha$-$CH_2CH_2$-$C\underline{H}_2$-OH], 6.50 [s, 1H, 4-C<u>H</u>], 6.55 [d, 1H, 2-C<u>H</u>] 7.05 [d, 1H, 1-C<u>H</u>].

FABMS (LSIMS): m/z: 444 [M^+] for $C_{27}H_{43}SiO_3$

Step D: <u>Synthesis of 3-tert-butyldimethylsilyloxy-17α-[propoxy-4-oxobutanoicacid]-17β-estradiol</u>:

At 0°C, Triethylamine (0.254 ml, 2.76mmoles) was added dropwise to a mixture of 3-tert butyldimethylsilyloxy-17α-[3-hydroxypropane]-17β-Estradiol (**III**, 0.410 mg, 0.92 mmoles, Succinic anhydride (92 mg, 0.92 mmoles), and DMAP (catalytic amount) in 5ml of dry DCM. The resulting mixture was continued stirring at room temperature for 12h under nitrogen. The reaction mixture was diluted with water (20ml), acidified with dilute HCl until the pH reaches between 3-4 as tested by litmus paper. The mixture was extracted with DCM (2x20ml) and the

organic layer was dried over anhydrous Na_2SO_4. After evaporating the organic layer the crude compound was purified by column chromatography with chloroform/methanol (98.5:1.5) as eluting solvent mixture to give 410 mg (81.6%) of Acid (**IV**).

^{1}H NMR (300 MHz, $CDCl_3$) **:** δ =0.19 [s, 6H, $Si(C\underline{H}_3)_2$], 0.92 [s, 3H, 18-C<u>H</u>3], 0.99 [s,9H, $SiC(C\underline{H}_3)_3$], 1.22-2.30 [m, 17H, cyclopentane, cyclohexance ring protons , 17α-$C\underline{H}_2C\underline{H}_2$-$CH_2$-O], 2.5-2.72 [m, 4H, 17$\alpha$ -OC(O)$C\underline{H}_2C\underline{H}_2$COOH], 2.80 [m, 2H, 6-$C\underline{H}_2$], 4.0-4.32 [dm, 2H,17$\alpha$ -$C\underline{H}_2$-OC(O)CH_2CH_2COOH], 6.49 [s, 1H, 4-C<u>H</u>], 6.55 [d, 1H, 2-C<u>H</u>] 7.05 [d, 1H, 1-C<u>H</u>].

FABMS (LSIMS): m/z: 544 [M^+] for $C_{31}H_{47}SiO_6$

Step E: <u>Synthesis of 17α-[propoxy-4-oxobutanoicacid]-17 β-Estradiol</u> :

To a stirred solution of 3-tert-butyldimethylsilyloxy-17α-[propoxy-4-oxobutanoicacid]-17β-Estradiol (**IV**, 100 mg, 0.183 mmole) dissolved in dry DCM (3 ml) was added (0.159 ml, 0.5514 mmole) tetra-n-butylammonium fluoride (1M solution in THF) drop-wise at 0^0 C under nitrogen. After addition the reaction was continued stirring at room temperature for 6h. The organic solvent mixture was evaporated and the crude product so obtained was purified by column chromatography with chloroform/methanol (97:3) as eluting solvent mixture to give 52 mg (66.6%) of TBS-deprotected acid (**V**).

^{1}H NMR (300 MHz, CD_3OD) **:** δ =0.9 [s, 3H, 18-C<u>H</u>3], 1.0-2.3 [m, 17H, cyclopentane, cyclohexance ring protons, 17α-C<u>H</u>$_2$C<u>H</u>$_2$-CH$_2$-O], 2.5-2.6 [m, 4H, 17α-OC(O)C<u>H</u>$_2$C<u>H</u>$_2$COOH], 2.80 [m, 2H, 6-C<u>H</u>$_2$], 4.1 [m, 2H, 17α-C<u>H</u>$_2$-OC(O)CH$_2$CH$_2$COOH], 6.49 [s, 1H, 4-C<u>H</u>], 6.55 [d, 1H, 2-C<u>H</u>] 7.05 [d, 1H, 1-C<u>H</u>].

ESIMS: m/z: 453 [M + 23] for $C_{35}H_{33}O_6$

Step F: Synthesis of DPPE-PEG-ES: A mixture of DPPE-PEG(2000)-NH_2 (60 mg, 0.0219 mmol) and17α-[propoxy-4-oxobutanoicacid]-17β-Estradiol (**V**, 37 mg, 0.0876 mmol) and DMAP (catalytic amount) were taken in 3ml dry DCM and stirred over ice bath. After 0.5 h DCC (18 mg, 0.0876 mmol) was added to it and continued stirring at the room temperature for 12 h. The solvent was evaporated and the crude product was purified three times by recrystalization using methanol-ether (1:15 v/v) as solvent. The purified compound obtained as a white gummy material (40 mg, 57.9% yield with respect to the PEG-lipid, Rf 0.1 at chloroform : methanol (90:10).

^{1}H NMR (300 MHz, $CDCl_3$, the representative peaks)**:** δ =0.90 [t, 6H, -OCO-CH$_2$-(CH$_2$)$_{14}$-CH$_2$-C<u>H</u>$_3$], 1.2-1.4 [m, 56H, -OCO-CH$_2$-(C<u>H</u>$_2$)$_{14}$-CH$_2$-CH$_3$], 6.50 [m, 1H, 4-C<u>H</u>], 6.65-6.85 [dd, 1H, 2-C<u>H</u>], 6.90-7.20 [dm, 1H, 1-C<u>H</u>].

The integration of the protons in the aromatic moiety when attached to the high molecular weight PEG does not give accurate peak heights in

contrast to other protons in NMR, as reported by us previously (ref. 18 in the main text). The mass of the product DSPE-PEG-ES is assumed to be 3150 as indicated by MALDI-TOFF mass spectra and as explained in text.

Liposome Preparation:

Preparation of PEG-lipid associated cationic liposomes: The lipid films were prepared by drying the chloroform solution of a total of 2.05 μmol of DODEAC, cholesterol, and DPPE-PEG-ES or DSPE-PEG-mal under a gentle stream of N_2 and dried in vacuum for at least 6h. The lipid mixtures were composed of DODEAC : Chol : DPPE-PEG-ES or DODEAC : Chol : DSPE-PEG-mal in a molar ratio of 1 : 1 : 0.05. It was hydrated with 1 ml of sterile water overnight and then first subjected to a low intensity bath sonication for 15 min at room temperature and then probe sonication for 2 min in ice using a constant duty cycle and output control magnitude of 2-3 in Branson Sonifier 450.

Size and surface charge measurements for liposomes and lipoplexes:

The sizes and the global surface charges (zeta potentials) of liposomes in plain DMEM and lipoplexes in plain DMEM or DMEM+7%FBS (a condition which mimics the lipoplex condition during transfection) were measured by photon correlation spectroscopy and electrophoretic mobility with a Zetasizer 3000HSA (Malvern Instruments, UK). The

system was calibrated by using the 199±6 nm Nanosphere™ Size Standard (Duke Scientific Corp., Palo Alto, CA, USA) and DTS 0050 standard from Malvern.

DNA Binding Assay:

The DNA binding ability of the targeted and non-targeted lipids containing DPPE-PEG-ES and DSPE-PEG-mal respectively was assessed by their gel retardation assay on a 0.8% agarose gel. 0.40 μg of pCMV-SPORT-βgal was complexed with the cationic lipids (at a cationic lipid: DNA charge ratio 8:1, 4:1, 2:1 and 1:1) in a total volume of 16 μl of HEPES buffer (pH 7.4) and incubated at room temperature for 30 min on a rotary shaker. 3 μl of 6X loading buffer (0.25% bromophenol blue, 40% sucrose) was added to it, and total solution was loaded to each well. The samples were electrophoresed at 80 V for approximately 2h and the DNA bands were visualized by staining for 30 min with ethidium bromide solution followed by 30 min destaining in water.

DNase 1 Sensitivity Assay:

In a typical assay, 3 nmol of DNA (1 μg) was complexed with both targeted and non-targeted cationic lipids containing DPPE-PEG-ES and DSPE-PEG-mal respectively in a (+/-) charge ratios of 8:1, 4:1, 2:1 and 1:1. The mixture was incubated at room temperature for 30 min on a

rotary shaker. Subsequently, the complex was treated with DNase-I (at a final concentration of 10 ng/3 nmol of pDNA) in the presence of 20 mM $MgCl_2$. The volume was made to 50 µl with HEPES buffer (pH 7.4) and incubated at 37^0C for 0.5 h. To stop the hydrolysis reaction EDTA was added to a final concentration of 20 mM and the mixture was incubated at 60^0C for 10 min in a water bath. The aqueous layer was extracted with 50 µl of phenol: chloroform mixture (1:1 v/v) and subsequently centrifuged at 10,000 rpm for 5 min. The aqueous supernatant was separated, loaded (20 µl) on a 0.8% agarose gel, and electrophoresed at 80 V for 3 h. DNase-I treated and untreated naked DNA was also included in the same experiment. The bands were visualized after 45 min ethidium bromide staining followed by 30 min destaining in water.

Gene Transfection

For β-gal transfection experiments, cells were seeded at a density of 15,000 (MDA-MB 231 Cells were seeded at a density of 12,000 & L-27 cells were 8,000) cells/well in a 96 well plate usually 18-24 h before transfection. 0.30 µg of pCMV-SPORT-β-gal DNA (diluted to 50 µl with plain DMEM) was complexed with varying amount of cationic liposomes (diluted to 50 µl with plain DMEM) for 30 min. The charge ratios (or, mole ratio for cationic lipid : DNA) were 8:1, 4:1, 2:1 and 1:1. After the complexation was completed, 200µl of DMEM containing

10% FBS (CM1X) were added to the resulting lipoplexes for triplicate experiments. Thus the final concentration of serum became 6.7%. Cells were washed with phosphate-buffered saline (PBS), pH 7.4 (1 x 200µl) and then with lipoplex (100 µl). After incubation of the cell plates at a humidified atmosphere containing 5% CO_2 at 37^0 C for 4 h, 100µl of DMEM containing 10% FBS (CM1X) were added to cells. The b-gal reporter gene activity was assayed after 48 h. The media were removed completely from the wells and cells were lysed with 50µl of 1X reporter lysis buffer (Promega, WI, USA) for 30 min. The β-galactosidase activity per well was estimated by adding 50 µl of 2X substrate (1.33 mg/ml of ONPG, 0.2 M sodium phosphate, pH 7.3 and 2 mM magnesium chloride) to the cell-lysate in the 96 well plate. Absorption of the product *ortho*-nitrophenol at 405 nm was converted to absolute β-galactosidase units using a calibration curve constructed with commercial β-galactosidase enzyme.

For the Tamoxifen pretreated experiments, cells (in 100µl of complete medium) were treated Tamoxifen (in Methanol) in a final concentration of 100 µM respectively for 1h. Cells were incubated at a humidified atmosphere containing 5% CO_2 at 37^0 C. Media were removed and the cells were washed with PBS (1 x 100µl). The cells were subsequently treated with the lipoplexes and reporter gene assay was performed according to the above-mentioned procedure.

For serum dependence study, the lipoplex upon incubation for 30 min in DMEM were added 200 µl of either of the following: a) DMEM b) 10% FBS containing DMEM media c) 20% FBS containing DMEM media d) 40% FBS containing DMEM media e) 80% FBS containing DMEM media f) 100% FBS. 100 µl of each of the 300 µl of resulting complex solution were added to each of the cell-well of the triplicate experiment and the cells were incubated for 4h as described above. The final percentages of serum will be 0, 7, 13, 26, 53, and 66 respectively during incubation. After 4h the media were removed and the cells were incubated in 10 % FBS containing DMEM media and continued incubating for 48h. The cellular assays were further done as described previously.

The transfection values are reported as the average values of triplicate experiment performed in the same plate on the same day. To verify reproducibility, each transfection experiment in MCF-7 cell was performed at least four times on four different days. Other cell line data were the representative data of at least two transfection experiments. The day to day variations in transfection efficiency were mostly within two- to three- folds and were dependent on the condition of cells.

X-Gal Staining:

Cells expressing β-galactosidase were histochemically stained with the substrate 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) as described previously (1). Briefly, forty eight hours after transfection with lipoplexes in 96 well plates, the cells were washed two times (2 x 100 μL) with phosphate-buffered saline (PBS, 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4, pH 7.4) and fixed with 0.5% glutaraldehyde in PBS (225 μL). After 15 minutes incubation at room temperature, the cells were washed again with PBS three times (3 x 250 μL) and subsequently, were stained with 1.0 mg/mL X-gal in PBS containing 5.0 mM $K_3[Fe(CN)_6]$, and 5.0 mM $K_4[Fe(CN)_6]$ and 1 mM $MgSO_4$ for 2-4 hours at 37° C. Blue colored cells were identified by light microscope (Leica, Germany). A minimum of 100 cells were counted to determine the percentage of cells expressing β-galactosidase.

p-53 Mediated Targeted Cytotoxicity

The MCF-7 cells were plated at a cell density of 5000 cells/well in 96 well plate usually 18-24 h before transfection. 0.30 μg of pCMV-p53 DNA (diluted to 50 μl with plain DMEM) was complexed with varying amount of cationic liposomes (diluted to 50 μl with plain DMEM) for 30 min. The cationic lipid to DNA charge ratios (or mole ratios) was maintained and the transfection protocol was completed following the earlier described β-gal transfection procedure. The cells were

maintained in 3 different 96-well plates and were tested for cell viability after 2, 4 and 6 days using MTT reduction assay as described earlier (2). Briefly, following completion of respective incubation period of 2, 4 and 6 days post p53 plasmid transfection, 10 µl of MTT (5 mg/ml in PBS) was added to each well and kept at 37^0 C for 2-3h. The medium was removed completely and the cells were dissolved in 50 µl of DMSO : methanol (50:50) and the UV absorption was measured at 550 nm. Absorbance of cells, untreated with either of the lipoplex, at 550 nm upon MTT treatment is depicted as A_{550}(untreated cells). Absorbance of cells, treated with either of the targeted or non-targeted lipoplex, at 550 nm upon MTT treatment is depicted as A_{550}(treated cells).

Results are expressed as:

Percent viability = [A_{550}(treated cells)-background]/[A_{550}(untreated cells)-background] X 100

Cell Viability assay:

Cytotoxicity of the cationic lipids was assessed using the MTT reduction assay as described above. The cytotoxicity assay was performed in 96 well plates by keeping the ratio of number of cells to amount of cationic lipid constant as is maintained in previously

described transfection experiments. The results are expressed in the equation as described above.

Statistical Analysis:

Data were expressed as mean ± standard derivation and statistically analyzed by the two-tailed unpaired Student t-test using the Microsoft Excel software program (Microsoft, Seattle, WA). For b-gal transfection experiments data were primarily considered significant if $p < 0.0001$, whereas for p-53 mediated targeted cell killing experiments data were considered significant if $p<0.005$.

Reference:

1. P. Schoen, A. Chonn, p. R. Cullis, J. Wilschut, P. Scherrer, *Gene Ther* **1999**, 6, 823-832.
2. M. B. Hansen, S. E. Neilson, K. Berg, *J. Immunol. Methods* **1989**, 119, 203-210.

ABBREVIATIONS

DODEAC: N,N-di-n-tetradecyl-N,N-(2-hydroxyethyl)ammonium chloride

Chol: Cholesterol

DSPE-PEG-mal: 1,2-Distearoyl-sn-glycero-phosphatidylethanolamine-[ω-maleimido-polyethylene glycol(2000)]

DPPE-PEG-NH_2: 1,2-Dipalmitoyl-*sn*-Glycero-3-Phosphoethanolamine-N-[Amino(Polyethylene Glycol)2000] (Ammonium Salt)

DPPE-PEG-ES: 1,2-Dipalmitoyl-sn-glycero-phosphatidylethanolamine-polyethylene glycol(2000)-ω–(17 β-Estradiol-17α-propoxy)-4-oxobutanamide.

DCC: Dicyclohexylcarbodiimide

DMAP: N, N-dimethylaminopyridine

ES: 17α-[propoxy-4-oxobutanoicacid]-17 β-Estradiol (modified estradiol)

PEG: Polyethylene glycol

MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

DMSO: Dimethyl sulfoxide

PBS: Phosphate buffered saline

X-gal: 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside

FBS: Fetal bovine serum

Supplementary Figure and Table Legends

Figure S1. Detailed synthetic scheme for the synthesis of DPPE-PEG-ES.

Figure S2. Mass spectra of compounds.

A. Electron spray mass spectra of estrogen fragment (17α-[propoxy-4-oxobutanoicacid]-17 β-Estradiol), which is reacted with DSPE-PEG-NH_2. The spectra gave a M+23 peak as described in materials and method section.

B. MALDI-TOF Mass spectra of DSPE-PEG-NH_2.

Figure S3. Results of gel retardation and Dnase I sensitivity assay.

A. DNA binding study. The pCMV-β-gal DNA is complexed with targeted and non-targeted cationic liposomes and subjected to gel electrophoresis. The targeted cationic lipid/DNA complex is depicted as DPPE-PEG-ES and the non-targeted control cationic lipid/DNA complex is depicted as DSPE-PEG-mal. Cationic lipid/ DNA complex with charge ratio varying from 1:1 to 8:1 is compared with naked pCMV-β-gal (shown in gel as DNA).

B. DNase-I treatment study. DNase-I treated targeted and non-targeted cationic lipid/pCMV-β-gal complexes with charge ratio varying from 1:1 to 8:1 are subjected to run in gel electrophoresis and simultaneously compared to naked pCMV-β-gal (shown in gel as DNA) and DNase-I treated naked pCMV-β-gal (shown in gel as DNA+ DNase-I). The targeted cationic lipid/DNA complex is depicted as DPPE-PEG-ES and the non-targeted control cationic lipid/DNA complex is depicted as DSPE-PEG-mal.

Figure S4. Serum stability of targeted lipid/DNA complex. MCF-7 cells were transfected with 0.3 μg pCMV-β-gal complexed in estradiol-associated targeted cationic liposome (DPPE-PEG-ES, black square) in the presence of 0, 7, 13, 26, 53 and 67% of serum in the transfection

media. The transgene expression obtained for each data point was acquired from triplicate treatments done in a single day.

Figure S5. Cellular toxicity of lipid/DNA complex. Different sets of MCF-7 cells were treated differently: a) some cells remained untreated b) some cells were treated with 0.3 μg of pCMV-β-gal complexed with estradiol-associated cationic liposome (DPPE-PEG-ES, white circle) C) some cells were treated with 0.3 μg of pCMV-β-gal complexed with non-targeted 'control' cationic liposome (DSPE-PEG-mal, black triangle). The lipid/DNA complexes were treated to the cells over the varied charge ratio (+/-) of 8:1, 4:1, 2:1 and 1:1. The viability of cells was measured immediately after the treatment by MTT assay.

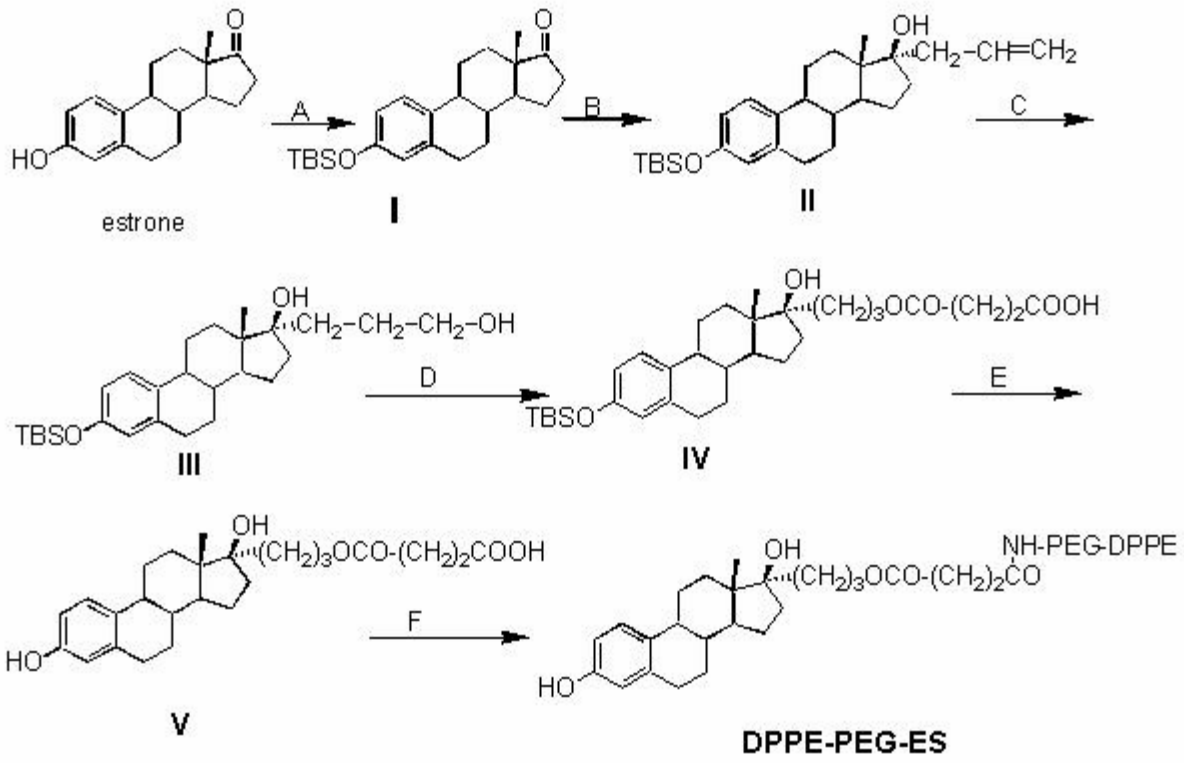
Figure S6. **Percentages of MCF-7 cells undergone transfection**. Histochemical X-gal staining of transfected MCF-7 cells with lipoplexes containing either DPPE-PEG-ES or DSPE-PEG-mal at lipid:DNA charge ratios of 8:1, 4:1, 2:1 and 1:1. Cells expressing β-galactosidase were stained with X-gal as described in the text. A minimum of 100 cells were counted to estimate the percent of cells transfected. The values shown are average of three independent measurements.

Figure S7. β-gal gene expressions in T47D cells. T47D cells with or without 100 μM tamoxifen pretreatment were transfected with 0.3 μg of pCMV-β-gal DNA complexed in estradiol-associated targeted cationic liposome (DPPE-PEG-ES) or non-targeted control cationic liposome (DSPE-PEG-mal). Cells are all treated in presence of 7% serum. The Transgene expression is represented as β-galactosidase (milliunits) activity. The targeted and non-targeted lipoplex were treated to the cells in cationic lipid to DNA charge ratios (+/-) 8:1, 4:1, 2:1, and 1:1. The transgene expression obtained for each data point was acquired from triplicate treatments done in a single day. The data were represented as following: a) cells treated with targeted lipoplex containing DPPE-PEG-ES in the absence of the pretreatment of tamoxifen (DPPE-PEG-ES, *white bar*). b) cells treated with targeted lipoplex containing DPPE-

PEG-ES in the with the pretreatment of tamoxifen (DPPE-PEG-ES + 100 μM tamoxifen, *black bar*). c) cells treated with non-targeted lipoplex containing DSPE-PEG-mal in the absence of the pretreatment of tamoxifen (DSPE-PEG-mal, *gray bar*). d) cells treated with non-targeted lipoplex containing DSPE-PEG-mal with the pretreatment of tamoxifen (DSPE-PEG-mal + 100μM tamoxifen, *red bar*). The differences in the data obtained between 'DPPE-PEG-ES' and 'DSPE-PEG-mal' or 'DPPE-PEG-ES' and 'DPPE-PEG-ES + 100μM tamoxifen' are represented either as * or **. * =$p<0.02$ and ** = $p<0.005$

Table 1. Size and surface charge measurements for liposomes and lipoplexes. Sizes and Zeta potentials of liposomes (Table 1A) & lipoplexes made in plain DMEM (Table 1B) and lipoplexes in DMEM + 7 % FBS (Table 1C) were measured by laser light scattering. Values shown are the averages of three for size determination and ten measurements for zeta potential determination.

Figure S1



A. Tert-butyl dimethylsilyl trifluoromethane sulphonate (TBDMS triflate), 2,6-lutidine, dry DCM

B. Mg, Allyl bromide, dry THF, 1,2-dibromoethane (as initiator)

C. Borane dimethylsulfide complex (BH_3.DMS), 3N NaOH, 30% H_2O_2, dry THF

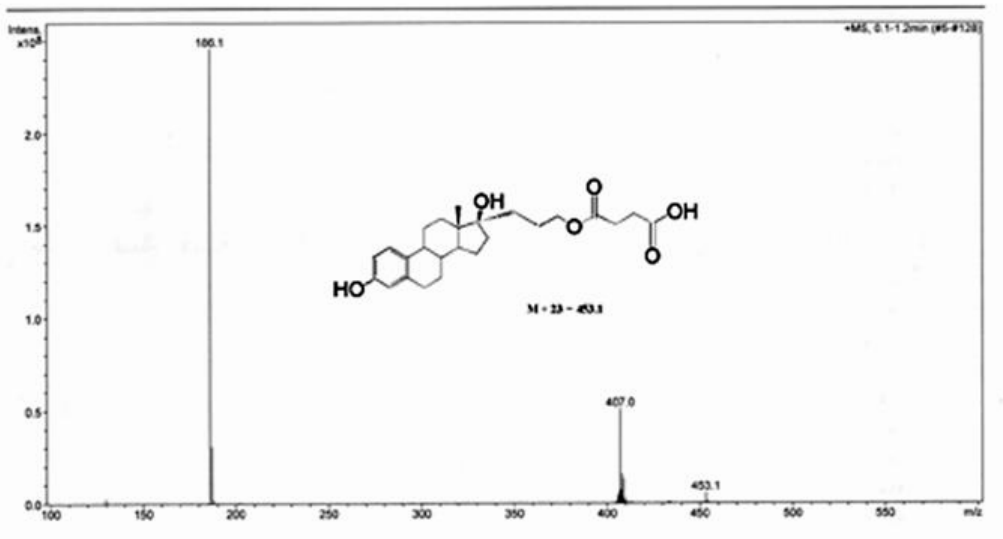
D. Succinic anhydride, triethylamine, DMAP, dry DCM

E. Tetra-n-butyl ammonium fluoride (TBAF), dry DCM

F. DPPE-PEG-amine, DCC, DMAP, dry DCM + dry DMF

Figure S2

A


100.1
407.0
453.1
OH
O
OH
O
HO
M + 23 = 453.1
m/z

B

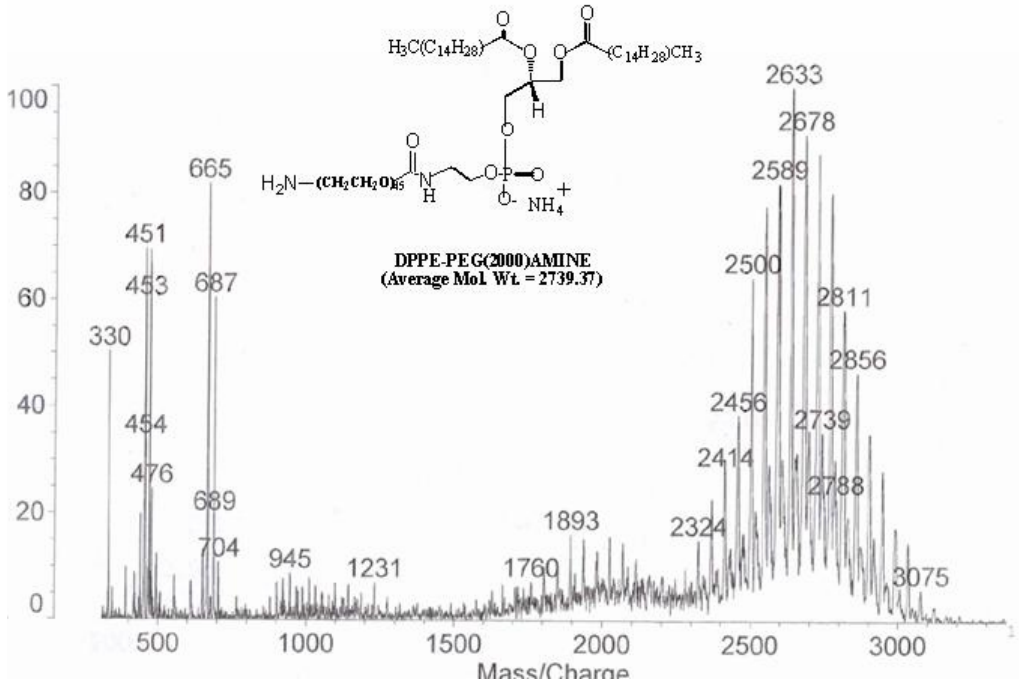
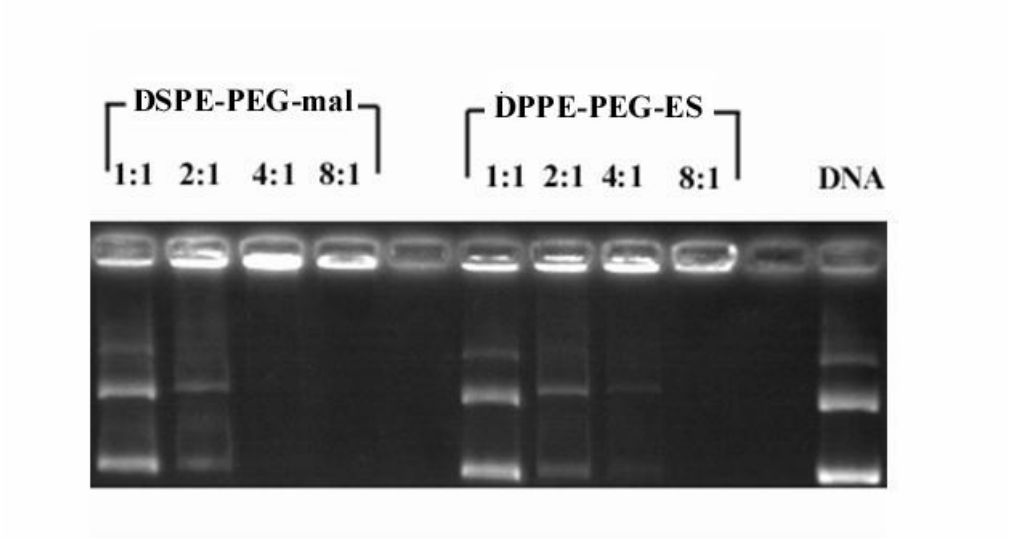

DPPE-PEG(2000)AMINE
(Average Mol Wt. = 2739.37)
100
80
60
40
20
0
500
1000
1500
2000
2500
3000
Mass/Charge
330
451
453
454
476
665
687
689
704
945
1231
1760
1893
2324
2414
2456
2500
2589
2633
2678
2739
2788
2811
2856
3075

Figure S3

A.


DSPE-PEG-mal
1:1 2:1 4:1 8:1
DPPE-PEG-ES
1:1 2:1 4:1 8:1
DNA

B.

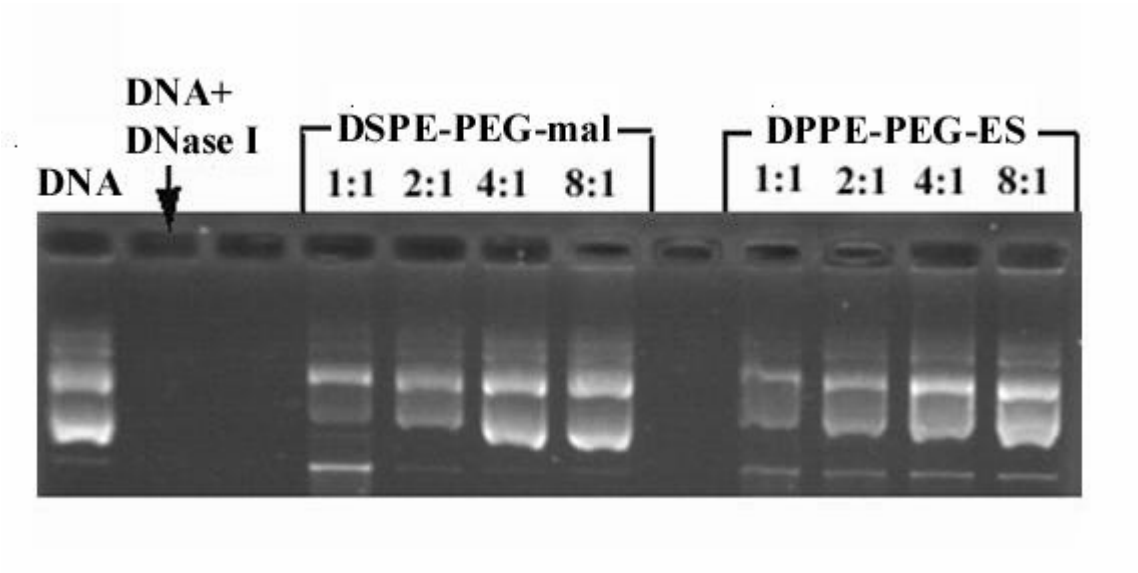

DNA+
DNase I
DNA
DSPE-PEG-mal
1:1 2:1 4:1 8:1
DPPE-PEG-ES
1:1 2:1 4:1 8:1

Figure S4.

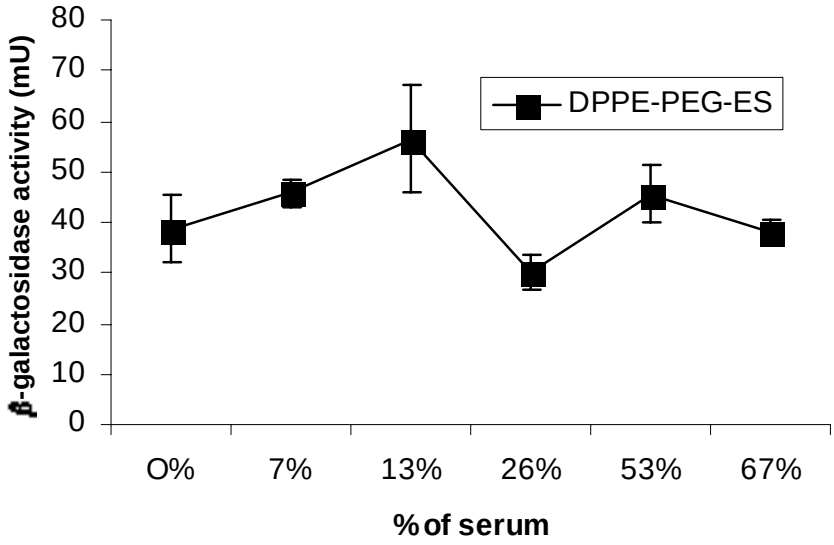


Figure S5.

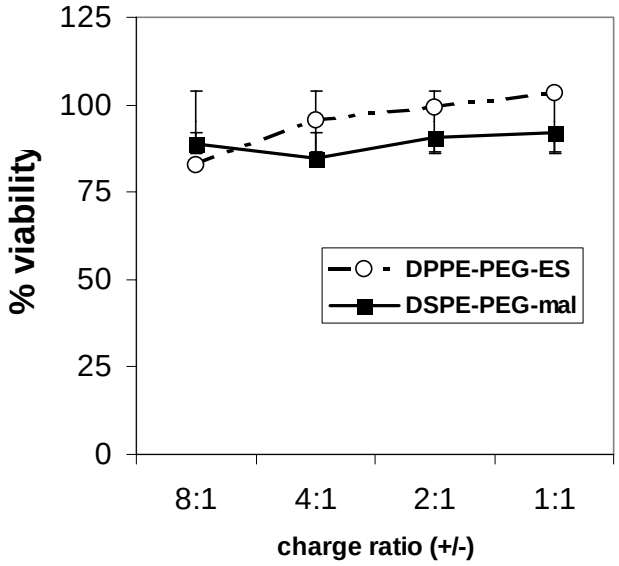


Figure S6

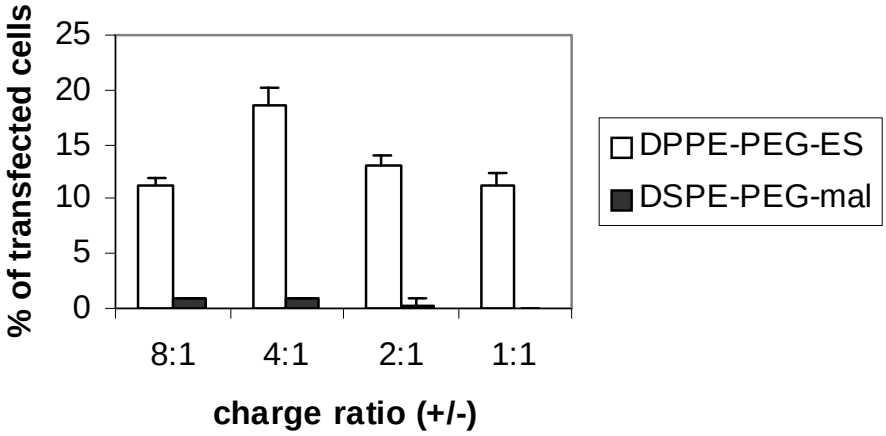


Figure S7

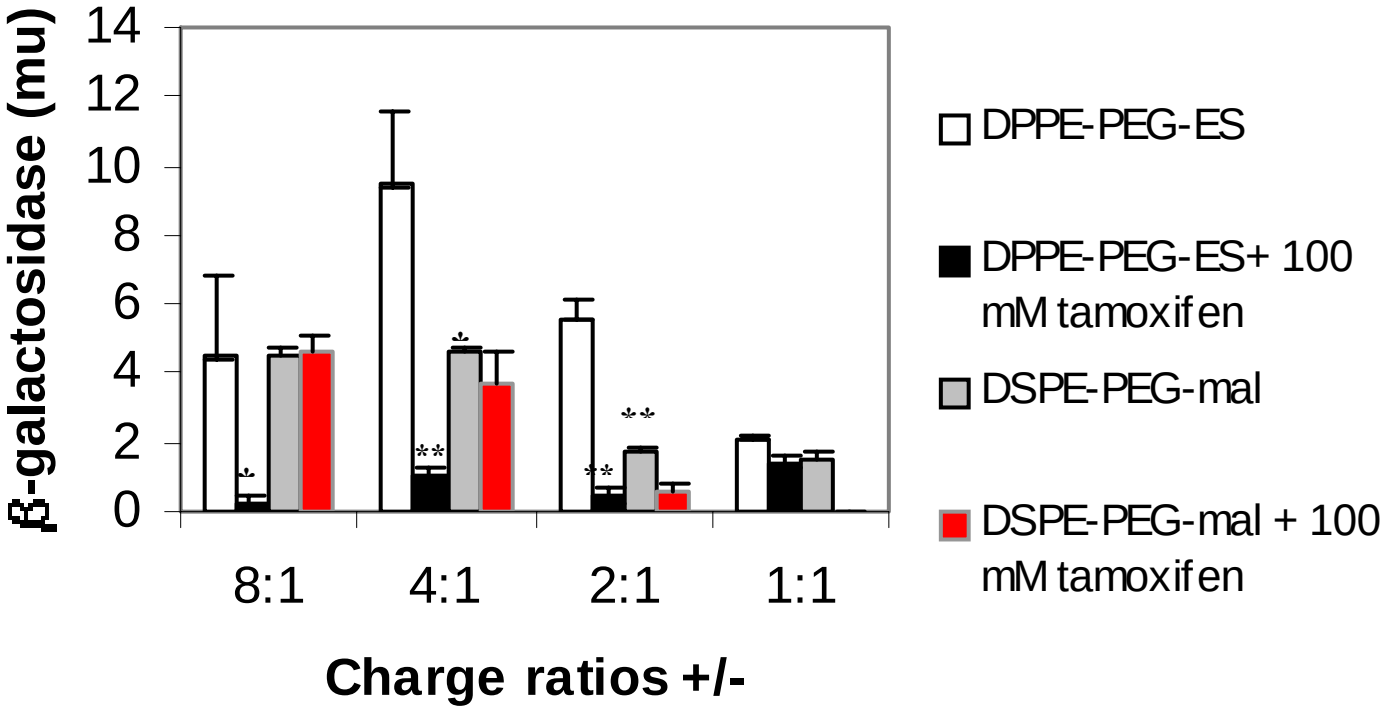


Table 1

A

Liposomes containing Lipids	**Sizes**	**Zeta potentials**
DPPE-PEG-ES	1415.6 ± 320.6	-1.4 ± 0.8
DSPE-PEG-mal	160 ± 27.7	-3.7 ± 1.3

B

Sizes of Lipoplexes in different charge ratios				
Liposomes containing Lipids	Lipid:DNA 8 :1	Lipid:DNA 4 :1	Lipid:DNA 2 :1	Lipid:DNA 1 :1
DPPE-PEG-ES	3418.1 ± 176.6	4418.5 ± 453.09	581.7 ± 26.6	1119.6 ± 172.7
DSPE-PEG-mal	138.6 ± 3.1	172.4 ± 2.9	165.2 ±21.0	234.2 ±33.3
Zeta potentials of Lipoplexes				
DPPE-PEG-ES	-9.1 ± 4.5	-14.2 ± 5.4	-11.3 ± 2.9	-18.4 ± 3.3
DSPE-PEG-mal	-2.1 ± 1.1	-5.4 ± 1.5	-5.1 ± 2.1	-5.2 ±2.5

C

Sizes of Lipoplexes in different charge ratios				
Liposomes containing Lipids	Lipid:DNA 8 :1	Lipid:DNA 4 :1	Lipid:DNA 2 :1	Lipid:DNA 1 :1
DPPE-PEG-ES	386 ± 25.93	134.7 ± 5.7	152 ± 27.5	128.5 ± 2.8
DSPE-PEG-mal	200.3 ± 5.3	147.3 ± 1.1	146.3 ± 2.1	150.3 ± 1.0
Zeta potentials of Lipoplexes				
DPPE-PEG-ES	-10.7 ± 3.6	-15.4 ± 2.7	-15.3 ± 2.2	-15.5 ± 2.7
DSPE-PEG-mal	-8.5 ± 3.9	-6.7 ± 1.4	-11.6 ± 1.6	-13.1 ±1.8