

Supporting Information

New pH-responsive Gemini Lipid Derived Co-liposomes for Efficacious Doxorubicin Delivery to Drug Resistant Cancer Cells

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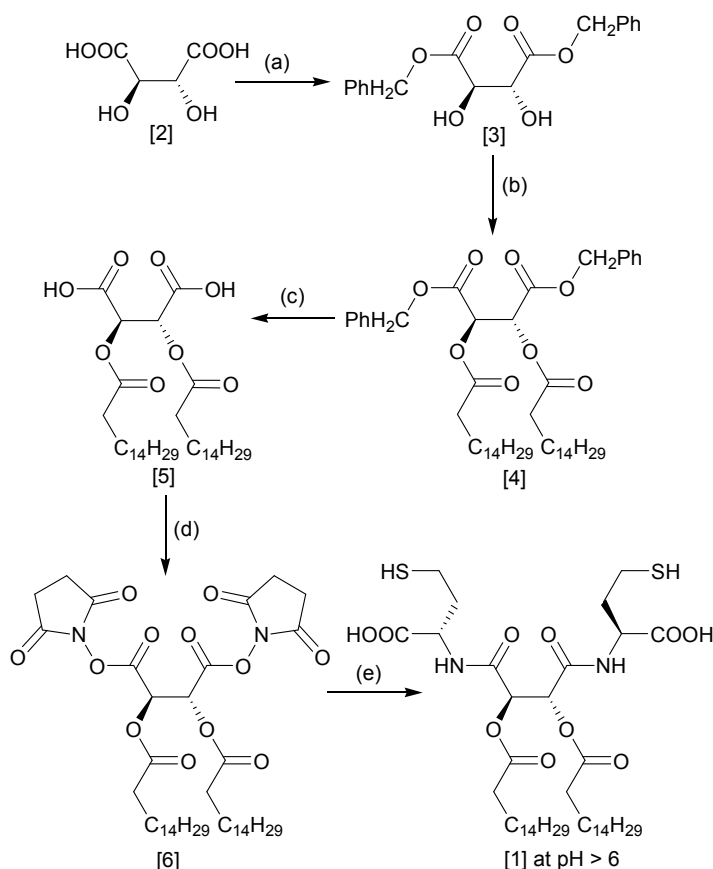
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1. Experimental Section

1.1. Materials and Methods. All reagents, solvents and chemicals used in this study were obtained from the best known commercial resources. The solvents were dried prior to use. Column chromatography was performed using a 60-120 mesh silica gel. NMR spectra were recorded using a Bruker-400 Avance NMR spectrometer (400 MHz for ^1H NMR). The chemical shifts (δ) are reported in ppm downfield from the internal standard, TMS, for ^1H NMR. Mass spectra were recorded on a MicroMass ESI-TOF spectrometer. Infrared (IR) spectra were recorded on a Jasco FT-IR 410 spectrometer. Compounds **3**,¹ **4**² and **5**² were synthesized according to reported procedures.

1.2. Synthesis.

Scheme S1. Synthesis of the gemini version of palmitoyl homocysteine (GPHC) based fusogenic lipid, **1**^a



^aReagents, conditions and yields: (a) PhCH_2OH , benzene, p-TsOH (Cat. amount), reflux, 24 h, quantitative yield; (b) $\text{C}_{15}\text{H}_{31}\text{COCl}$, CHCl_3 , Et_3N , DMAP (Cat. amount), $0\text{ }^\circ\text{C}$, 1 h, 87% yield; (c) Pd/C/H_2 (60 psi), EtOAc , rt, 2h, quantitative yield; (d) DCC, HOSU, dry EtOAc , DMAP (Cat. amount), rt, 16 h, 90% yield; (e) Homocysteine, sodium carbonate buffer of pH 9, rt, 16 h, 40% yield.

1.2.1. Synthesis of N-Hydroxysuccinimide Ester of Compound 5 (6).³ The compound **5** (1 g, 1.6 mmol) was added to a solution of N-hydroxysuccinimide (0.4 g, 3.5 mmol) in dry ethyl acetate (25 mL). A solution of DCC (0.72 g, 3.5 mmol) in dry ethyl acetate (10 mL) was then added, and the reaction mixture was left stirred overnight at room temperature. The reaction mixture was filtered and the filtrate was concentrated under reduced pressure to yield white solid. The crude material was then recrystallized from ethanol to yield pure N-hydroxysuccinimide ester of compound **5** (**6**, 1.1 gm, 84% yield). FT- IR (neat) (cm^{-1}): 2919, 2850, 1738, 1539, 1268, 1228, 1210, 1065, 1028, 806, 721. ^1H NMR (CDCl_3 , 400 MHz): δ (ppm): 0.87 (*t*, $J = 6.0$ Hz, 6H), 1.25 (*m*, 48H), 1.64 (*m*, 4H), 2.01 (*m*, 4H), 2.82 (*br*, 8H), 6.55-6.62 (*m*, 2H). ESI-MS: m/z calcd for ($M + \text{Na}^+$): 843.4983, found 843.4987.

1.2.2. Synthesis of Gemini version of Palmitoyl Homocysteine (GPHC) (1).⁴ Portions from two stock solutions of compound **6** (1 gm, 1.2 mmol) in 20 mL of THF and homocysteine hydrochloride (584 mg, 3.8 mmol) in 20 mL of distilled water were added alternately into a stirred mixture of 40 mL of 0.2 M Na_2CO_3 - NaHCO_3 buffer, pH 9.0 and 40 mL of THF. The mixture was stirred at ambient temperature for 16 h and then acidified with 1 M HCl to pH 2-3. Then the mixture was warmed to 60 °C and bubbled with N_2 to facilitate the precipitation of the thiolactone form of the compound. The product was collected and washed extensively with water. Recrystallization from methanol yielded pure compound **1** (787 mg, 75% yield). FT- IR (neat) (cm^{-1}): 2918, 2850, 1652, 1532, 1228, 1210, 1154, 1125, 963, 721, 562. ^1H NMR (CDCl_3 , 400 MHz): δ (ppm): 0.87 (*t*, $J = 6.0$ Hz, 6H), 1.23 (*m*, 48H), 1.64 (*m*, 4H), 1.98 (*m*, 4H), 2.38 (*t*, $J = 7.2$ Hz, 4H), 2.55 (*t*, $J = 7.2$ Hz, 4H), 4.52 (*m*, 1H), 4.68 (*m*, 1H), 6.55-6.62 (*m*, 2H). ^{13}C -NMR (CDCl_3 , 400 MHz): δ : 14.06, 22.63, 25.56, 29.30, 29.36, 29.63, 31.86, 57.2, 80.1, 173.1, 174.5. ESI-MS: m/z calcd for ($M + \text{Na}^+$): 883.5152, found 883.5154. Elem. anal.: calcd. for $\text{C}_{44}\text{H}_{80}\text{N}_2\text{O}_{10}\text{S}_2$: C, 61.36; H, 9.36; N, 3.25; Found: C, 61.5; H, 9.56; N, 3.37.

1.3. Preparation of LUVs. LUVs were prepared from mixtures of DOPE, cholesterol and palmitoyl homocysteine (PHC) or GPHC (compound **1**). In all the formulations the total lipid concentration was kept fixed at 0.5 mM. During the preparation of liposomes, cholesterol or PHC or GPHC were added at varying molar ratios w.r.t. DOPE. First lipids were dissolved in appropriate organic solvents, such as chloroform and methanol. This solution was then dried to a thin film under a stream of nitrogen gas. The resulting thin films were placed under high vacuum for at least 6 h to remove the last traces of the residual organic solvent. Then the lipid films were hydrated by vortex mixing in 1.0 ml of aqueous buffer containing 10 mM HEPES and 150 mM NaCl (pH 8.1). After hydration the multi-lamellar vesicles (MLVs) were subjected to alternative five freeze-thaw cycles. The MLV suspensions were then extruded to produce LUVs.

1.4. Dynamic Light Scattering (DLS) and Zeta Potential Measurements.⁵ DLS and zeta potential measurements were performed at room temperature using a Malvern Zetasizer Nano ZS particle sizer (Malvern Instruments Inc., MA). Samples were prepared and examined under dust-free conditions. Mean diameters reported here were obtained from a Gaussian analysis of the intensity-weighted particle size distributions.

1.5. UV-Visible Spectroscopy. The UV-Vis spectra of doxorubicin (DOX) for loading and release study were recorded on a Shimadzu model 2100 spectrophotometer.

1.6. Encapsulation of Doxorubicin. The lipid films were hydrated in a solution of doxorubicin hydrochloride, DOX (stock concentration 1 mg/mL) in PBS 1X buffer (pH 7.4). Lipid suspensions were vortexed and incubated for 48 hrs with intermittent vortexing. The sample was then centrifuged at 13000 rpm for 1 h at 25 °C in a Remi Superspin centrifuge and a residue was pelleted at the bottom. From the supernatant a small volume was taken and was examined under UV-Visible spectroscopy to measure the DOX content. The percentage encapsulation in vesicles was calculated using the following formula: encapsulation (%) = (absorbance of bound molecules/absorbance of total molecules) × 100. Here, the absorbance of bound molecules = (absorbance of total molecules – absorbance of supernatant molecules).

1.7. pH Triggered Release of Doxorubicin. The drug loaded vesicles were separated from the free drug by centrifugation at 13000 rpm for 1h. Then the pellets were resuspended into 1 mL of phosphate buffered saline of pH ~7.4 or phosphate buffer of pH ~6 or acetate buffer of pH ~5 and the release of DOX from the nanovesicles was followed by UV-visible spectroscopy at different time intervals. The percentage release was calculated using the following formula: release (%) = [{(Absorbance (t) / Absorbance (t₀)) - 1} × 100], where t was the time at which the absorbance was measured and t₀ was the initial time.

1.8. Atomic Force Microscopy (AFM) Imaging. AFM images were obtained by JPK instruments using NanoWizard JPK00901 software in the tapping mode. Analyses of the AFM images were processed using JPK data analyzer software. A sheet of freshly cleaved mica with the pre-air dried aqueous solution of the co-liposomes was glued over a plate using a very small piece of double-sided tape. The images in tapping mode were recorded using an NSC35/Si3N4/Al BS AFM tip with a resonance frequency between 150-300 kHz and force constants ranging from 5 to 40 Nm⁻¹.

1.9. Transmission Electron Microscopy (TEM) imaging. Aqueous suspensions of the co-liposomes were examined under transmission electron microscopy by negative staining using 0.5% uranyl acetate. 20 µL of each suspension was loaded onto a Formvar-coated, 400 mesh copper grid and allowed to remain for 15 min. Excess fluid was removed from the grid and 10 µL of 0.5% uranyl acetate was applied on the same grid. The grid was air-dried and then the last traces of solvent were removed under high vacuum. The samples were observed under TEM (TECNAI T20) operating at an acceleration voltage (DC voltage) of 100 keV. Micrographs were recorded at a magnification of 10000-80000 X.

1.10. Cell Culture. The drug sensitive epidermoid carcinoma cell, KB-3-1, colchicine resistant epidermoid carcinoma cell, KB-CH^R-8.5 and DOX resistant adenocarcinoma cell (HeLa-DOX^R) were cultured in Eagle's minimum essential medium containing 10% fetal bovine serum (FBS) and antibiotic, pen strep (100 units/mL penicillin and 100 µg/mL streptomycin). On the other hand, the non-cancerous fibroblast cell line, NIH3T3, was cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS) and antibiotic, pen strep (100 units/mL penicillin and 100 µg/mL streptomycin). Cells were incubated at 37 °C in humidified atmosphere (relative humidity >95%) with a CO₂ level of 5%. Trypsinization using 0.5% trypsin–EDTA was followed for passaging of cells at regular intervals.

1.11. Cytotoxicity Assay. Cytotoxicity of DOPE:cholesterol:GPHC co-liposomes at 16:16:1 molar ratio (with or without DOX) and free DOX were evaluated in NIH3T3, KB-3-1, KB-CH^R-8.5 and HeLa-DOX^R cell line by conventional 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay. In a typical experiment, cells were seeded at the density 10000 cells/well in a 96 well microplate. After 24 h, cells were treated with the co-liposomes at different concentrations for 72 h. In a separate experiment, the treatment of cells with DOX-loaded co-liposomes and free DOX was also performed for the requisite time period at the various drug concentrations. This was followed by the replacement with 200 µL of fresh medium and incubation for another 4 h with MTT (20 µL, 5 mg/mL in DPBS). Finally, the whole medium was removed from the wells, DMSO (200 µL) was added and absorbance was read on a microplate reader (Epoch 2, BioTek). Experiments were performed in triplicates for each concentration and the results expressed are from three such independent experiments.

1.12. Flow Assisted Cell Sorting. Evaluation of internalization of free DOX and DOX-loaded co-liposomes was performed by FACS analysis. Cells were first seeded at the density of 60000/well in a 24 well plate. Cells were treated with free DOX and DOX-loaded co-liposomes when cells were at about 80% confluent. After the treatments for required drug concentration (17.2 µM) and required time points (4, 8 and 12 h), medium was removed and cells were properly washed with DPBS three times followed by trypsinization to harvest the cells. Determination of the fluorescence intensities of internalized DOX was performed using flow cytometry (BD FACS Calibur, BD Biosciences, USA). Cells without any treatment served as control. Analysis of flow cytometry data was performed using WinMDI software where ten thousand events were collected for analysis.

1.13. Confocal Microscopy. For visualization of the intracellular delivery of DOX, HeLa-DOX^R cells were cultured on cover slips placed in 12-well cell culture plates. Drug treatment was performed using required drug concentration (34.5 µM) for 24 h. After treatment, cells were washed with DPBS buffer three times and fixed in 4% para-formaldehyde solution for 10 min. Cells were then rinsed again with DPBS buffer thrice and incubated with nuclear staining dye DAPI (4',6-diamidino-2-phenylindole) for 10 min. Wells were washed properly again to remove excess dye and control overstaining. The glass cover slips were taken out and mounted on glass

slides over ProLong Gold antifade reagent (molecular probes) and viewed under confocal laser scanning microscope (LSM meta, Zeiss).

1.14. Statistical Significance. Statistical significance of differences between control (free DOX) and samples (DOX loaded co-liposomes) were evaluated using two-way ANOVA using GraphPad Prizm 5.0 according to the applicability of the analysis. Results were considered statistically significant when the p value was less than 0.05 and denoted as either * $P < 0.05$, ** $P < 0.01$ or *** $P < 0.001$.

2. Figures

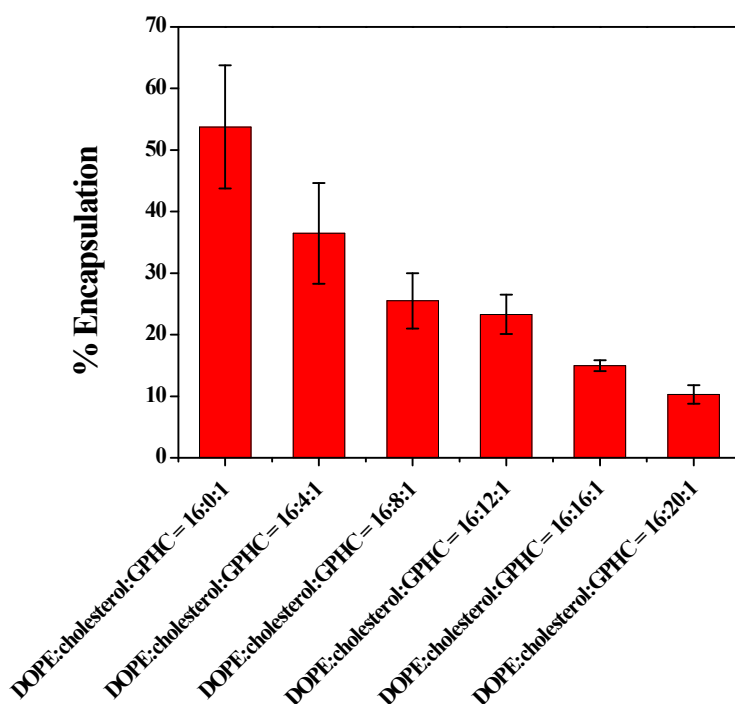


Fig. S1 Comparison of % encapsulation efficiency of DOX in cholesterol doped DOPE:GPHC (16:1) co-liposomes at different molar ratios.

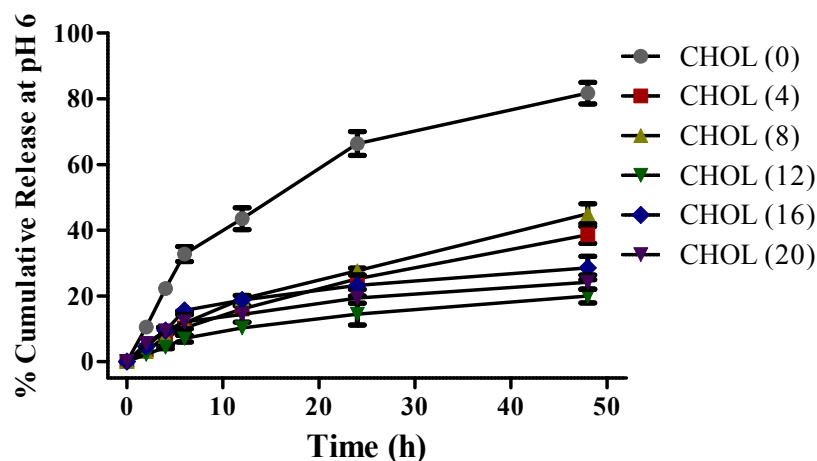


Fig. S2 Drug release kinetic profiles of cholesterol doped DOPE:GPHC (16:1) co-liposomes at various molar ratio at pH 6.0 for 50 h. The molar ratio of cholesterol mixed with the DOPE:GPHC (16:1) co-liposomes was mentioned in parentheses next to it.

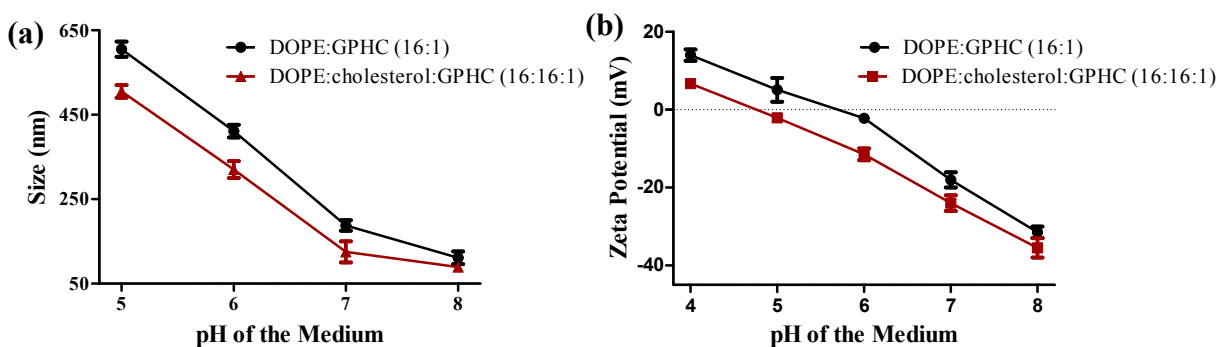


Fig. S3 (a) Size distribution and (b) zeta potential measurements of the most optimal co-liposomal formulation, DOPE:cholesterol:GPHC at 16:16:1 molar ratio, in comparison with its parent co-liposomal formulation, DOPE:GPHC at 16:1 molar ratio, at different pHs.

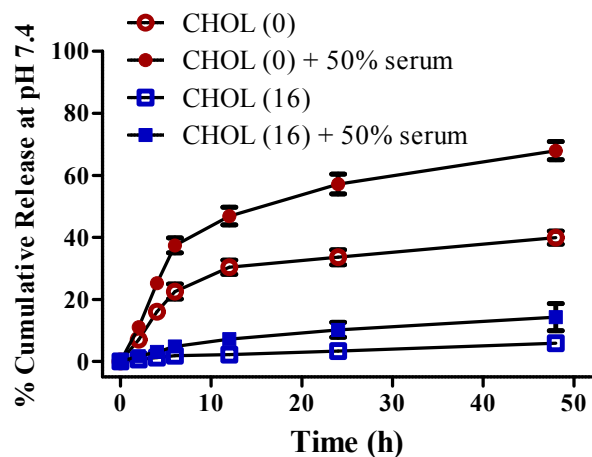


Fig. S4 Drug release kinetic profiles of the cholesterol doped DOPE:GPHC (16:1) co-liposomes at various percentage of cholesterol in 50% serum containing medium having pH~7.4 for continuous 50 h. The molar ratio of cholesterol mixed with the DOPE:GPHC (16:1) co-liposomes was mentioned in parentheses next to it.

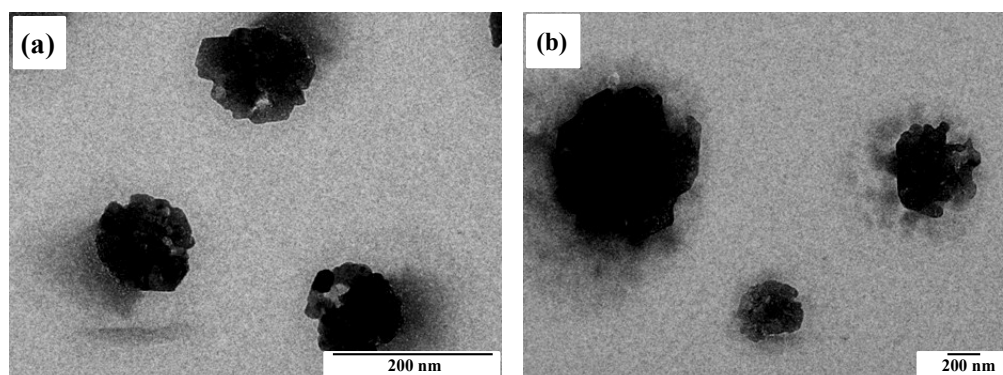


Fig. S5 Transmission electron microscopy (TEM) images of the most optimal co-liposomal formulation, DOPE:cholesterol:GPHC at 16:16:1 molar ratio, at (a) pH 7.4 and (b) pH 5.

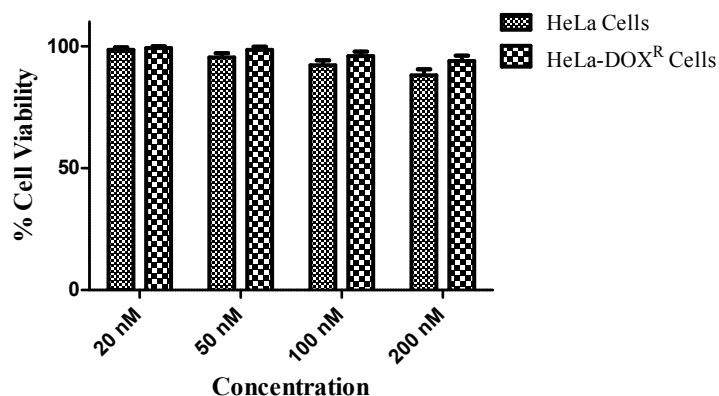


Fig. S6 Cytotoxicity of the co-liposomal formulation, DOPE:cholesterol:GPHC at 16:16:1 molar ratio, alone at different concentrations towards the drug-sensitive and drug-resistant cancer cell line.

2. References

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