Supporting Information

Mannose-6-Phosphate Functionalized Liposomal Nanocarrier for Lysosome-specific Delivery of β-Glucuronidase Enzyme in Drosophila Model of MPS VII

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Materials and methods

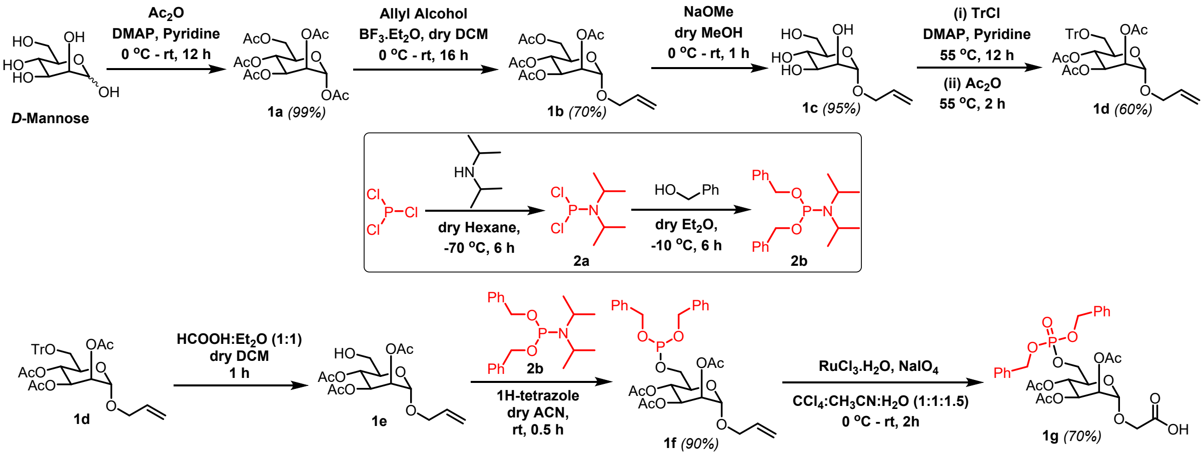
Unless otherwise noted, materials were obtained from commercial suppliers and were used without further purification. Unless otherwise reported, all reactions were performed under an argon atmosphere. Removal of solvent in vacuo refers to distillation using a rotary evaporator attached to an efficient vacuum pump. Products obtained as solids or syrups were dried under high vacuum.

All commercially available chemicals used in this study were purchased from Merck, India. D-Mannose, $BF_3.Et_2O$, Formic acid, Cholesterol, Trifluoroacetic acid (TFA), and 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) were purchased from SRL chemicals, India. 9-BBN dimer, Hexamethylenediamine, 1-bromooctadecane, Trityl chloride, Ruthenium chloride hydrate, Palladium charcoal (Pd/C), Proton sponge, Fl-NHS ester, Calcein, Rhodamine B-octadecyl ester (RBOE), Bovine serum albumin (BSA), 1,2-Di-O-hexadecyl-sn-glycero-phosphocholine, Sepharose CL-6B (cat no. CL6B200), and β-glucuronidase (bovine liver, CAS No. 9001-45-0, cat No. G0251-1MU) were purchased from Sigma Aldrich. Sodium methoxide was purchased from TCI Chemicals, India. Acetic anhydride was purchased from S D Fine-Chem Limited. L-lysine mono hydrochloride, Allyl alcohol, EDC.HCl, HOBT, N-Ethyldiisopropylamine (DIPEA), PCl_3, Diisopropylamine, and Boc anhydride were purchased from Spectrochem, India. Dialysis tubing (molecular weight cut-off, MWCO = 3.5 kDa) was purchased from Thermo Fisher Scientific. 4-Methylumbelliferyl-β-D-glucuronide (4-MUG, cat No. 44064) was purchased from Glycosynth. Sephadex G-25 medium (cat no. 17003301) and Track-Etch membrane (0.1 µm, 19 mm, used for extrusion, cat no. 10419504) were purchased from Cytiva. Ammonia solution, Hydrazine hydrate, Sodium metaperiodate, Benzyl alcohol, Triethylamine, Na_2HPO_4, NaH_2PO_4, Hexane, Ethyl acetate, Diethyl ether, Chloroform, Dichloromethane (DCM), Tetrahydrofuran (THF), N,N-Dimethylformamide (DMF), Acetonitrile, and Methanol were purchased from Merck, India. Solvents were dried over CaH_2 or Na in a distillation setup for use in the reaction. Analytical thin-layer chromatography was performed on pre-coated silica plates from Merck, India. All column chromatography purifications were performed using 100-200 mesh silica gel, unless otherwise noted. Solvents were evaporated by a rotary evaporator purchased from Buchi. All polymerization reactions were carried out in a Glovebox under an argon atmosphere. ^1H and ^{13}C NMR were performed on Jeol (400 MHz) and Bruker NMR Spectrometers (400 or 500 MHz). The NMR spectra were reported relative to the deuterated solvent used and processed using MestReNova software. Dynamic Light scattering measurements were performed on a Malvern Zetasizer Nano ZS. Transmission electron microscopy (TEM) measurements were performed at 200 kV using a JEOL-1200 instrument, and size analysis was conducted with ImageJ software. Atomic force microscopy (AFM) analysis was performed using an Asylum MFP-3D instrument, and the images were analyzed with WSxM software. For the MTT assay, the reading was taken at 570 nm wavelength in an ELISA Synergy H1 Microplate reader instrument. A Leica SP8 confocal microscope, equipped with a 63X oil immersion objective, was used for all imaging. Images were analyzed using the LASx software.

Experimental section

Synthesis of M6P-carboxylic acid

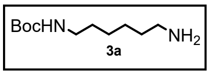
The compound was synthesized by modifying our previously reported methodology to some extent.[1]



Scheme S1: Synthetic scheme of preparation of M6P functionalized carboxylic acid.

Procedure for the synthesis of *N*-6-aminohexyl-*N,N*-di-n-octadecylamine (3c)

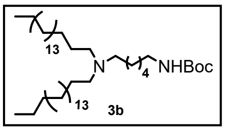
a. Preparation of *N*-Boc-1,6-hexanediamine (3a)



Hexamethylenediamine (2.2 gm, 18.9 mmol) was dissolved in 40 mL MeOH in a 100 mL RB, and di-tert-butyl pyrocarbonate (1.0 gm, 4.7 mmol) in 20 mL MeOH was added at 0 °C to it in a dropwise manner over 3 hours. The reaction was stirred at RT for 12 hours, and after completion, the solvent was evaporated to dryness. Further, purification was done using workup with water (100 mL) and extracted with diethyl ether thrice (3 x 150 mL). Finally, the organic layer was dried over anhydrous MgSO4 to isolate the pure product (1.05 gm, 25% yield) as a yellow solid.

^{1}H NMR (500 MHz, $CDCl_3$) δ 1.27 (s, 5H), 1.38 (s, 18H), 2.62 (t, 2H), 3.04 (s, 2H), 4.64 (s, 1H). **^{13}C NMR (126 MHz, $CDCl_3$)** δ 26.58, 26.67, 28.48, 30.10, 33.73, 40.55, 42.14, 78.98, 156.08.

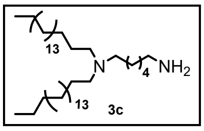
b. Preparation of *N*-Boc-*N,N*-dialkyl-1,6-hexanediamine (3b)



The synthesized N-tertbutyloxycarbonyl-6-aminohexylamine (1.08 gm, 5 mmoles) was dissolved in dry EtOAc (50 mL). Activated K2CO3 (3.43 gm, 25 mmol) and 1-bromooctadecane (4.17 g, 12.5 mmol) were added under argon and refluxed for 36 hours. After completion, the solvent was evaporated to full dryness, and then the reaction mixture was solubilized in 150 mL chloroform, washed with water (3 x 150 mL), dried over anhydrous MgSO$_4$, and filtered. After removing chloroform from the filtrate, silica gel column chromatographic purification of the resulting residue (using 60-120 mesh silica gel size and 30% EtOAc in hexane, as the eluent) afforded pure product as a light yellow brown solid (1.38 gm, 50% yield).

^{1}H NMR (500 MHz, CDCl$_3$) δ 0.86 (t, 6H), 1.24 (s, 70H), 1.42 (s, 20H), 2.35 (t, 6H), 3.09 (s, 2H), 4.53 (s, 1H). **^{13}C NMR (126 MHz, CDCl$_3$)** δ 14.22, 22.81, 27.08, 27.46, 27.82, 28.55, 29.49, 29.79, 29.83, 32.05, 40.69, 40.70, 54.26, 54.35, 79.09, 156.12.

c. Preparation of *N*-6-aminohexyl-*N,N*-di-n-octadecylamine (3c)



The synthesized tert-butyl (6-(hexyl(octadecyl)amino)hexyl)carbamate (1.38 gm, 2.5 mmol) was dissolved in DCM (10 mL), and trifluoroacetic acid (TFA) (5 mL) was added to it at 0 °C. The resulting solution was stirred at room temperature overnight to ensure complete deprotection. Excess TFA was removed by flushing nitrogen to give the title compound as a trifluoroacetate salt. Further, the compound was dissolved in chloroform (100 mL) and washed with aqueous saturated NaHCO3 (3 x 100 mL) and brine (100 mL). The organic layer was dried over anhydrous MgSO4, filtered, and the solvent from the filtrate removed by rotary evaporation afforded pure free amine of N-6-aminohexyl-N,N-di-n-octadecylamine as a light yellow solid (1.07 gm, 95% yield).

^{1}H NMR (500 MHz, CDCl$_3$) δ 0.87 (t, 6H), 1.25 (s, 64H), 1.41 (h, 8H), 1.65 (s, 2H), 2.37 (t, 6H), 2.67 (t, 2H). **^{13}C NMR (126 MHz, CDCl$_3$)** δ 14.24, 22.83, 27.04, 27.13, 27.19, 27.68, 27.83, 29.50, 29.80, 29.85, 32.07, 33.97, 42.34, 54.40. **HRMS (ESI-TOF):** m/z [M+H]$^+$: Calculated- 621.7026, Experimental- 621.7031.

DLS measurement

DLS measurements were performed on a ZetaSizer Nano series (Malvern Instruments) at 25 °C in quartz cuvettes. All the data were collected in triplicates, each averaged for 5 scans and averaged over to present the data. DLS of all the liposomal solutions were measured in water/phosphate buffer (20 mM at pH 7)/serum (10% FBS) containing DMEM media at 0.125 mg/mL concentration after filtering it through 0.22 µm nylon syringe filters.

Sample preparation for TEM

Firstly, the liposomal nanocarrier was formulated following the method provided in the Experimental section of the main manuscript, and 10 µL of solution (0.125 mg/mL) was drop-casted onto a 200-mesh copper carbon-coated grid and maintained for full adsorption. The grids were stained with 5 µL of uranyl acetate solution (2 mg mL^{-1}) for 5 seconds, excess solvent was soaked by Whatman filter paper, and then washed with DI water thrice to remove unbound uranyl acetate, followed by soaking in Whatman filter paper. Next, the grids were high-vacuum dried using a desiccator for a day. Images were recorded using a Joel-1200 TEM instrument working at 200 kV.

Sample preparation for AFM study

Firstly, the liposomal nanocarrier was formulated according to the method provided in the Experimental section, and 5 µL of the solution (0.125 mg/mL) was drop-casted onto a piece of silicon wafer and allowed to adsorb completely. Next, the wafers were vacuum-dried using a desiccator for one day. Images were recorded using an Asylum MFP-3D AFM instrument.

Degradation of LN in the esterase-rich medium

The formulated LN was incubated with esterase (Sigma Aldrich, cat no.) and kept on a shaker for 48 hours. In the meantime, DLS analysis was performed using the Zetasizer instrument. TEM and AFM samples were also prepared at specific time intervals, and analysis was conducted.

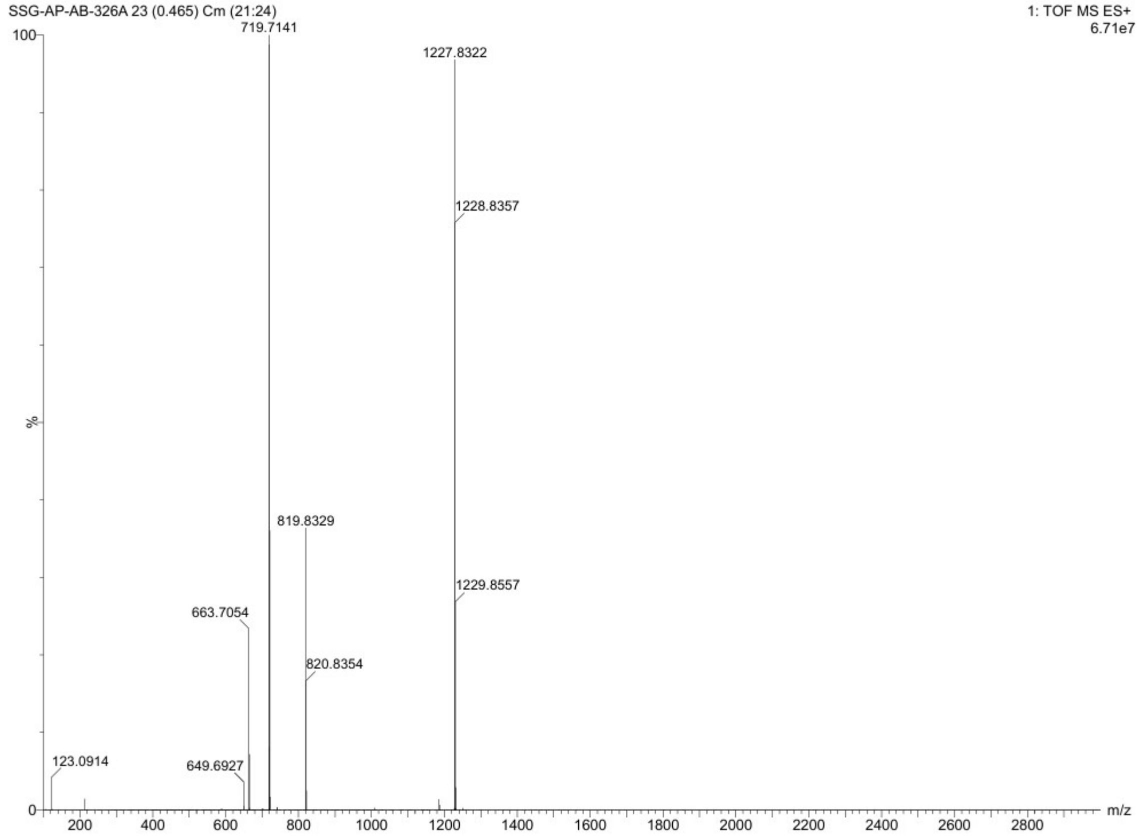


Figure S1: HR-MS of the protected **M6PGL**.

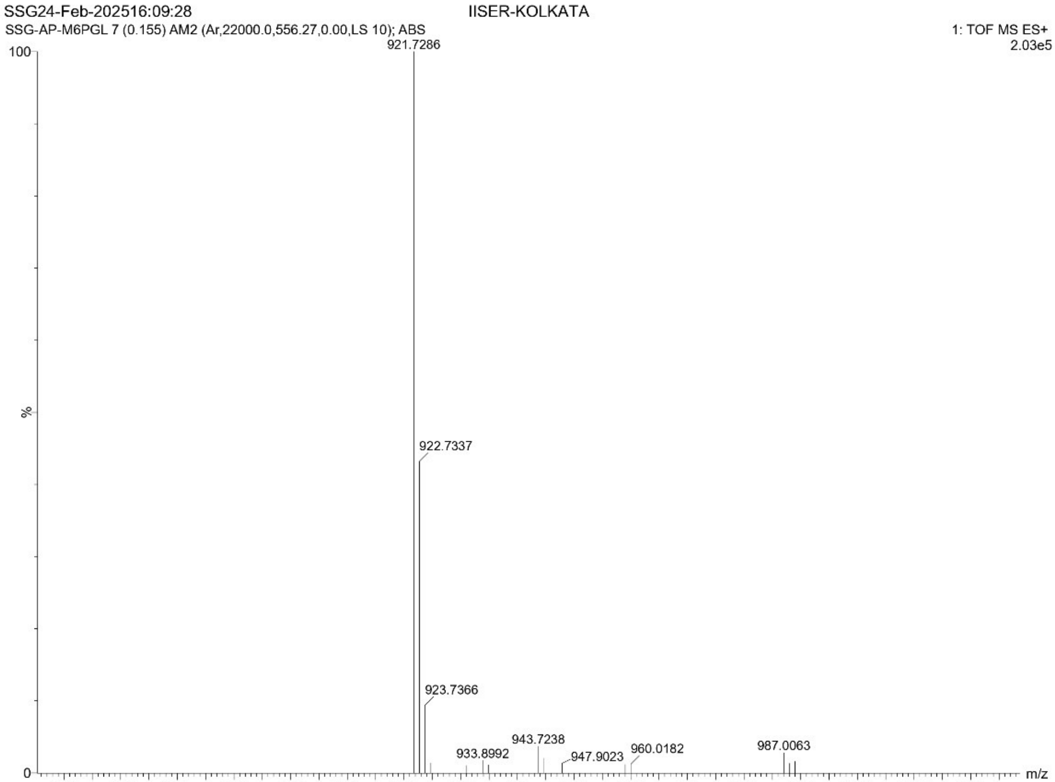


Figure S2: HR-MS of the deprotected **M6PGL**.

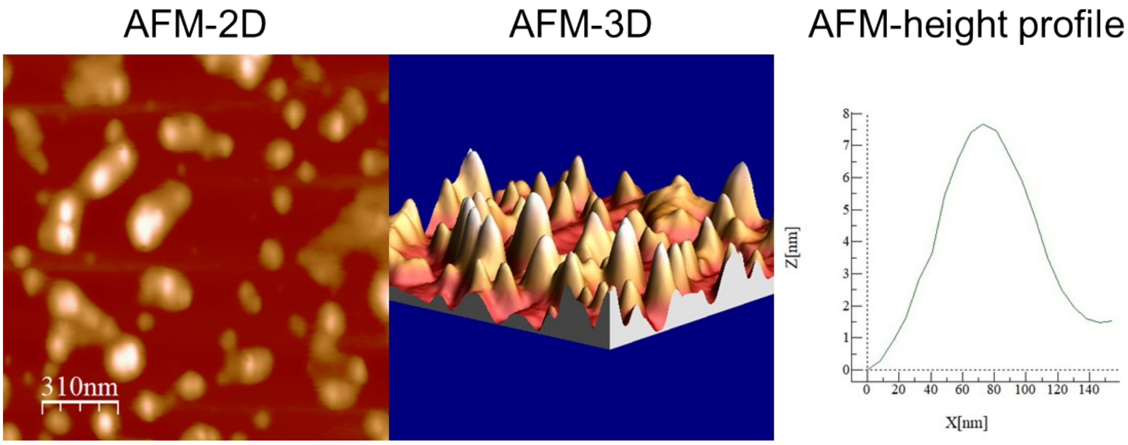
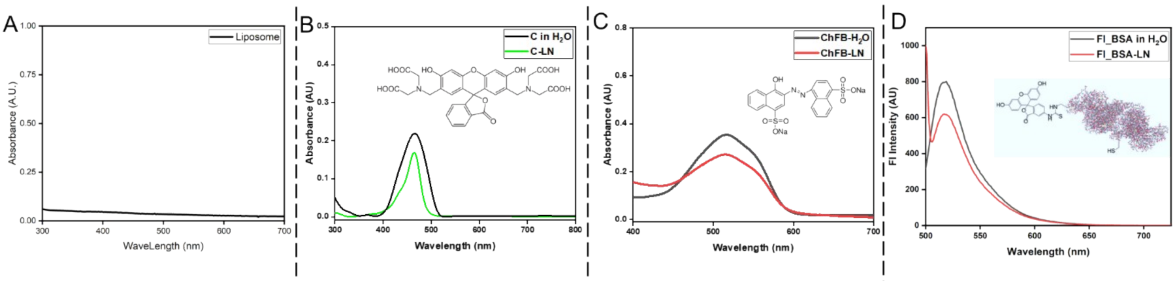


Figure S3: AFM analysis showing 2D, 3D images, and height profile of spherical **LN**.



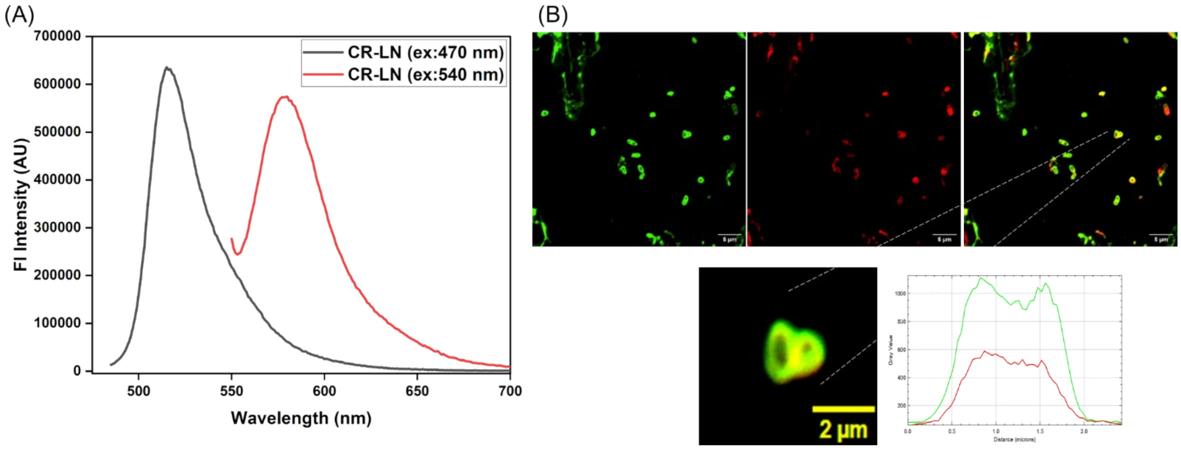
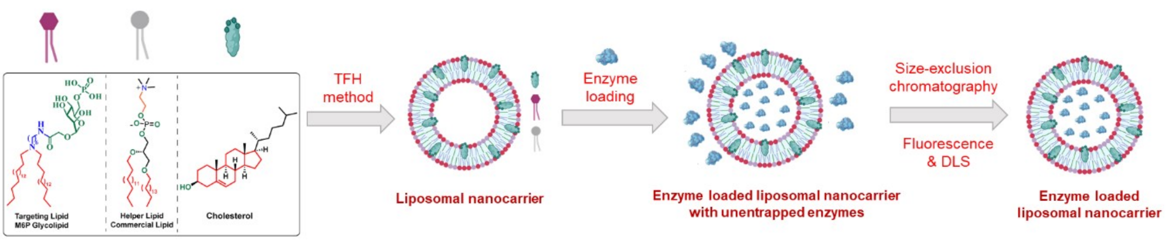


Figure S4: UV-Visible spectra of (A) **LN**, (B) **C-LN**, (C) **ChFB-LN**, Fluorescence spectra of (**Fl_BSA-LN**).

Figure S5: (A) Fluorescence spectroscopic studies of **CR-LN**, (B) Confocal microscopy analysis of **CR-LN** in which Calcein and RBOE were found colocalized.

Figure S6: Schematics of the formulation of protein-encapsulated **LN**.



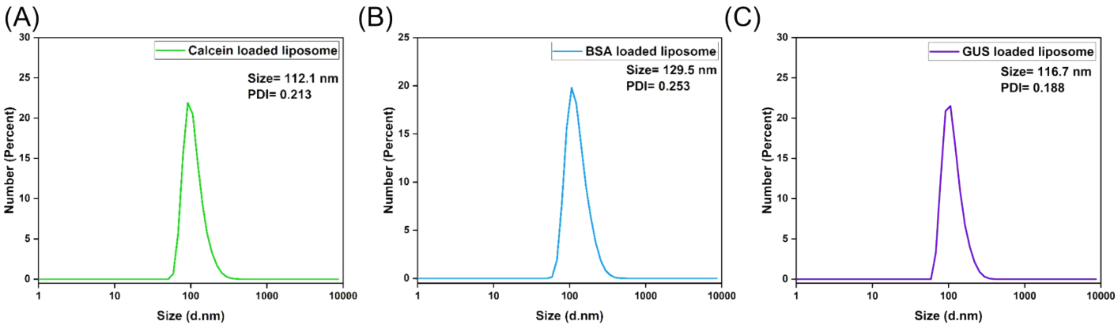


Figure S7: DLS analysis of dye/protein-loaded liposomal nanocarriers; (A) **C-LN**, (B) **BSA-LN**, (C) **GUS-LN**.

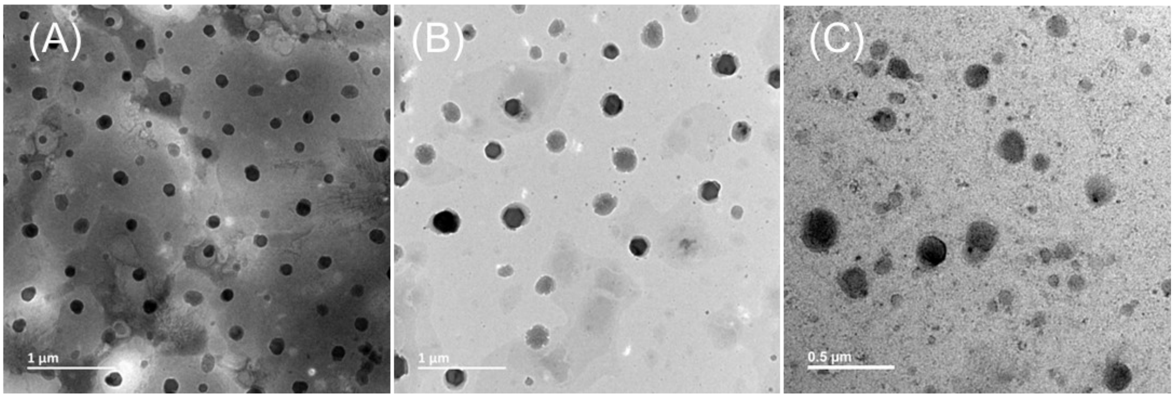


Figure S8: TEM analysis of dye/protein-loaded liposomal nanocarriers; (A) **C-LN**, (B) **BSA-LN**, (C) **GUS-LN**.

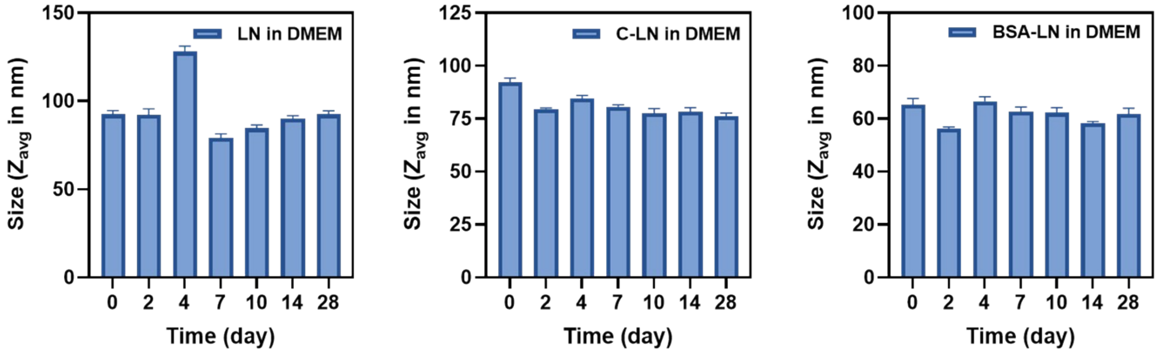


Figure S9: DLS stability analysis of dye/protein-loaded liposomal nanocarrier in serum (10% FBS) containing DMEM media (A) **LN in DMEM**, (B) **C-LN in DMEM**, (C) **BSA-LN in DMEM**.

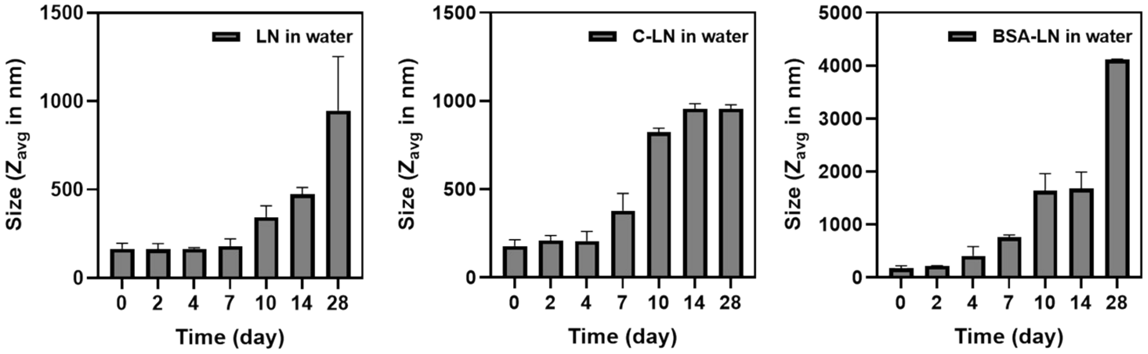
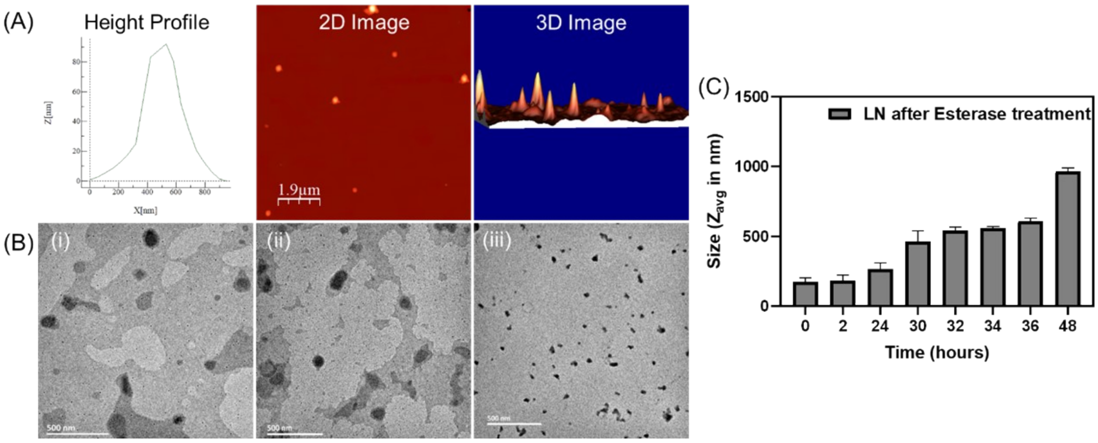


Figure S10: DLS stability analysis of dye/protein-loaded liposomal nanocarrier in water (A) **LN in water**,

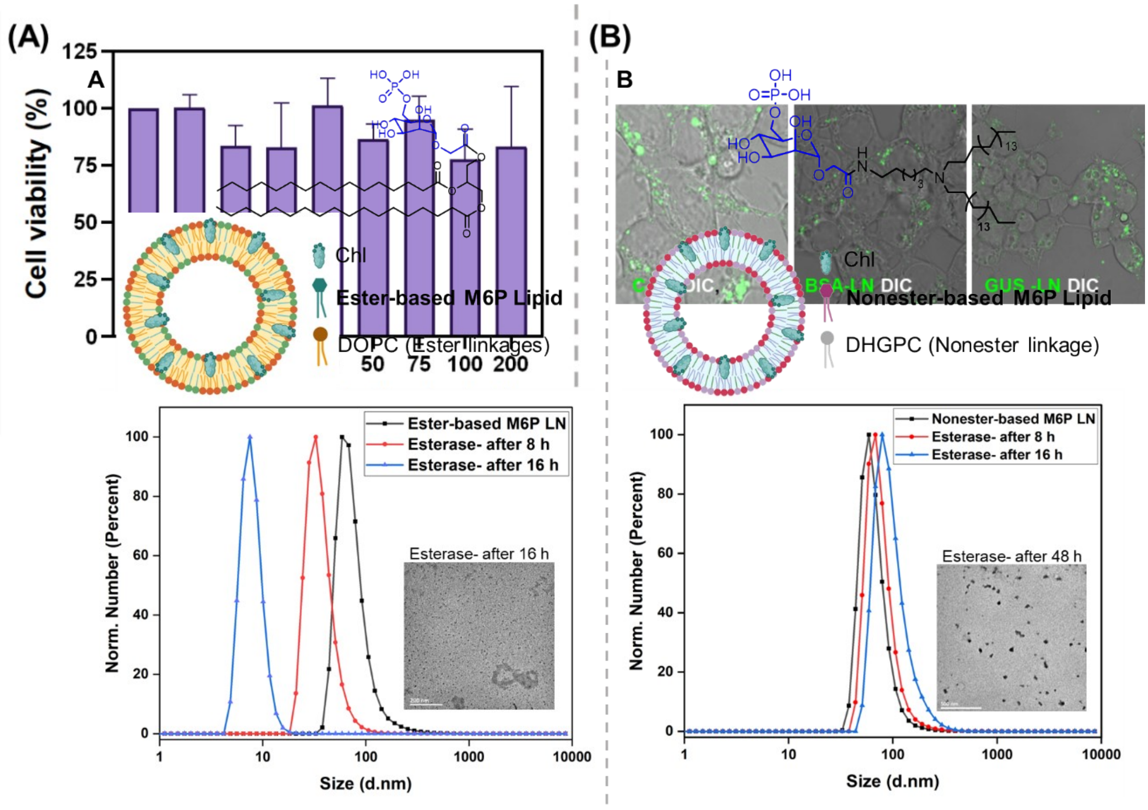


(B) **C-LN in water**, (C) **BSA-LN in water**.

Figure S11: Size and morphology analysis of **LN** after esterase treatment; (A) AFM images of esterase treated **LN** after 36 hours incubation, (B) TEM images of esterase treated **LN** after (i) 24 hours, (ii) 36 hours, (iii) 48 hours of incubation, (C) DLS analysis of **LN** at different time interval up to 48 hours of incubation.

Figure S12: (A) structure of ester-based M6P lipid (synthesized in previous work[2]), formulation of ester-based M6P **LN** and its DLS analysis after 8 h and 16 h incubation with esterase enzyme, TEM analysis after 16 h of incubation; (B) structure of nonester-based M6P lipid (synthesized in this work), formulation of nonester-based M6P **LN**, and its DLS analysis after 8 h and 16 h incubation with esterase enzyme, TEM analysis after 48 h of incubation.

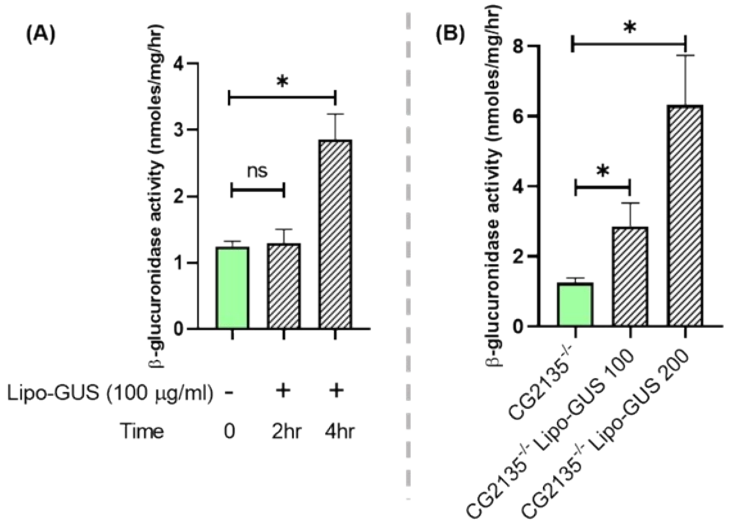
Discussion: In the AFM (A), TEM images (B-i & ii), the spherical morphology was intact after esterase treatment and incubation for 36 hours. The structures degraded to smaller fragments after 48 hours,



as seen in TEM images (B-iii). The abrupt high Z-average values were also observed at 48 hours, as indicated by DLS analysis.

Figure S13: (A) In vitro cytotoxicity (MTT) assay or cell viability test of the liposomal nanocarrier (**LN**) was done to determine the toxicity of HEK293 cells treated with **LN**s in different concentrations (µg/mL). The viability was compared to that of untreated cells. The bar graphs represent mean ± SEM (N=3); (B) dye/protein-loaded **LN** is uptaken by HEK293 cells; DIC images of HEK293 cells treated with **C-LN**, **BSA-LN**, and **GUS-LN** (green).

Figure S14: Time and dose-dependent enhancement in the β-GUS activity after internalization of **GUS-LN**. (A) Hemocytes were treated with **GUS-LN** at an effective GUS concentration of 100 µg/mL, incubated for 0 (untreated), 2, and 4 hours, and compared with untreated cells, showing a time-dependent increase in enzyme activity. N=3. (B) Hemocytes treated with **GUS-LN** at an effective GUS concentration of 100 and 200 µg/mL were incubated for 4 hours and compared with untreated cells, showing an increment in enzyme activity with increasing dose from 100 to 200 µg/mL. Enzyme activity is represented as nmololes of 4-MU produced per hour per mg, N=3. Error bars represent mean±SEM. Asterisks denote a level of significance, p≤0.05*, Student's t-test.



^{1}H, ^{13}C, ^{31}P NMR spectra of the compounds

(* peak indicates residual solvent peak)

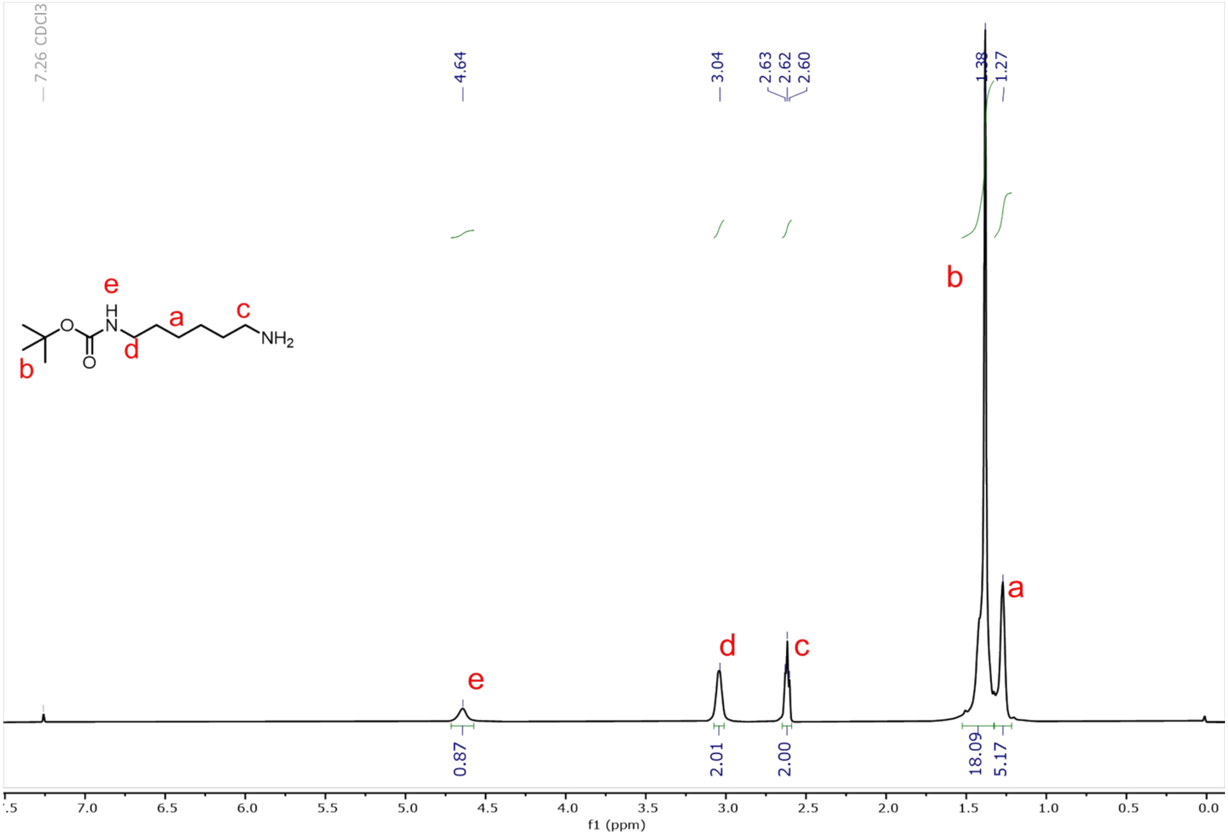


Figure S15: ^{1}H NMR of N-Boc-1,6-hexanediamine.

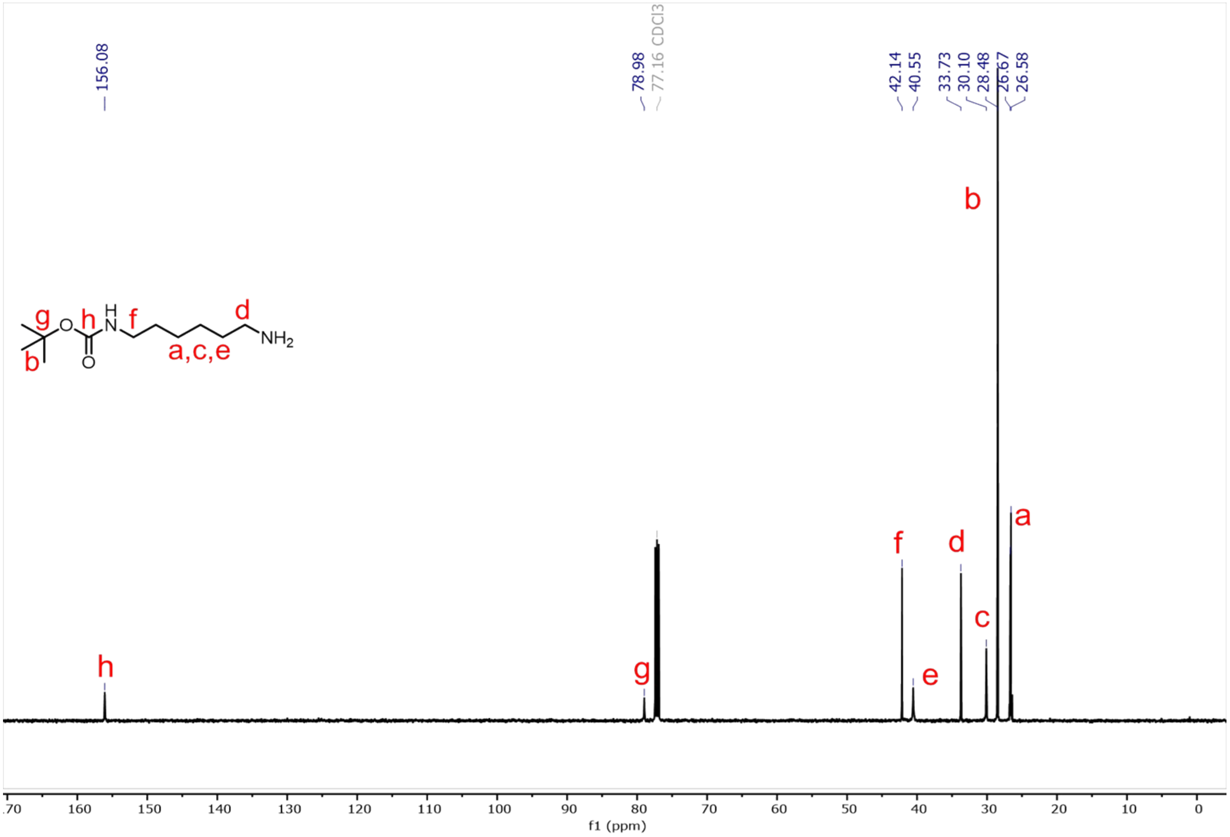


Figure S16: ^{13}C NMR of N-Boc-1,6-hexanediamine.

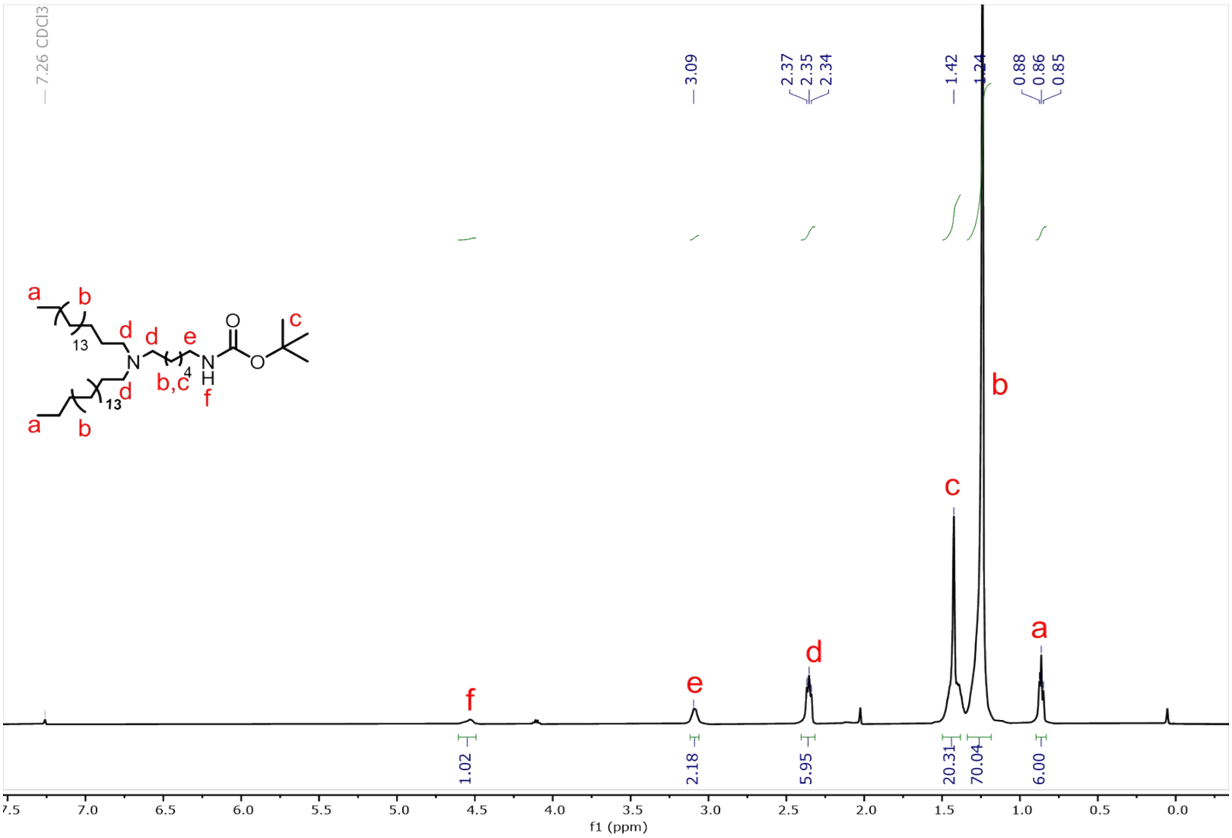


Figure S17: ^{1}H NMR of N-Boc-N,N-dialkyl-1,6-hexanediamine.

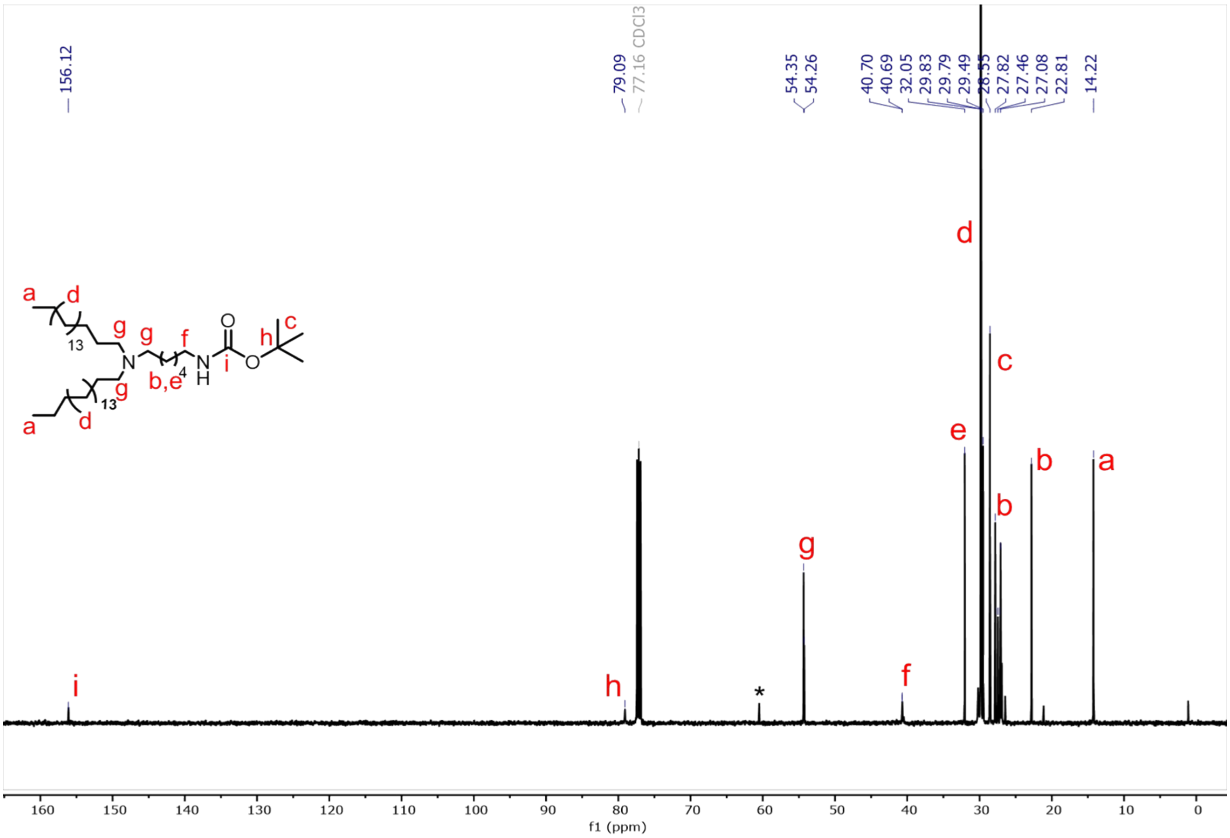


Figure S18: ^{13}C NMR of N-Boc-N,N-dialkyl-1,6-hexanediamine.

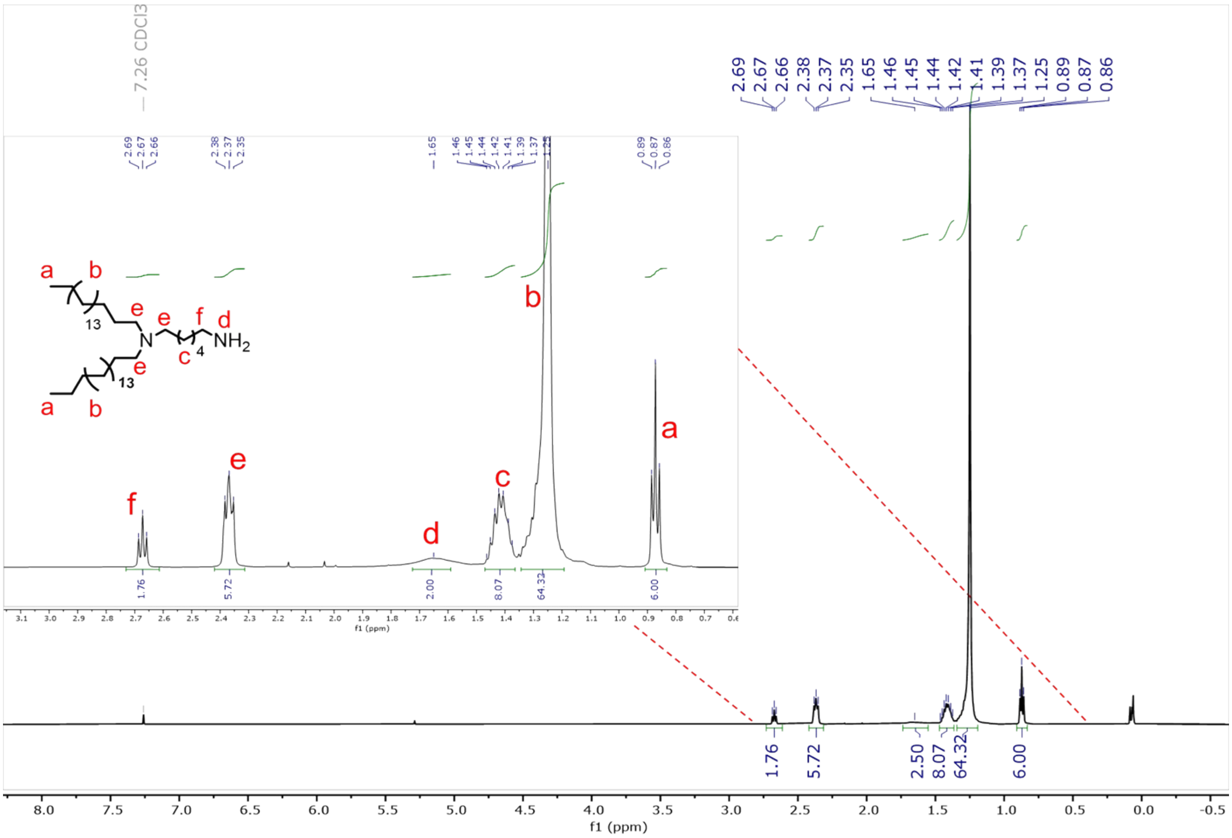


Figure S19: ^{1}H NMR of N-6-aminohexyl-N,N-di-n-octadecylamine

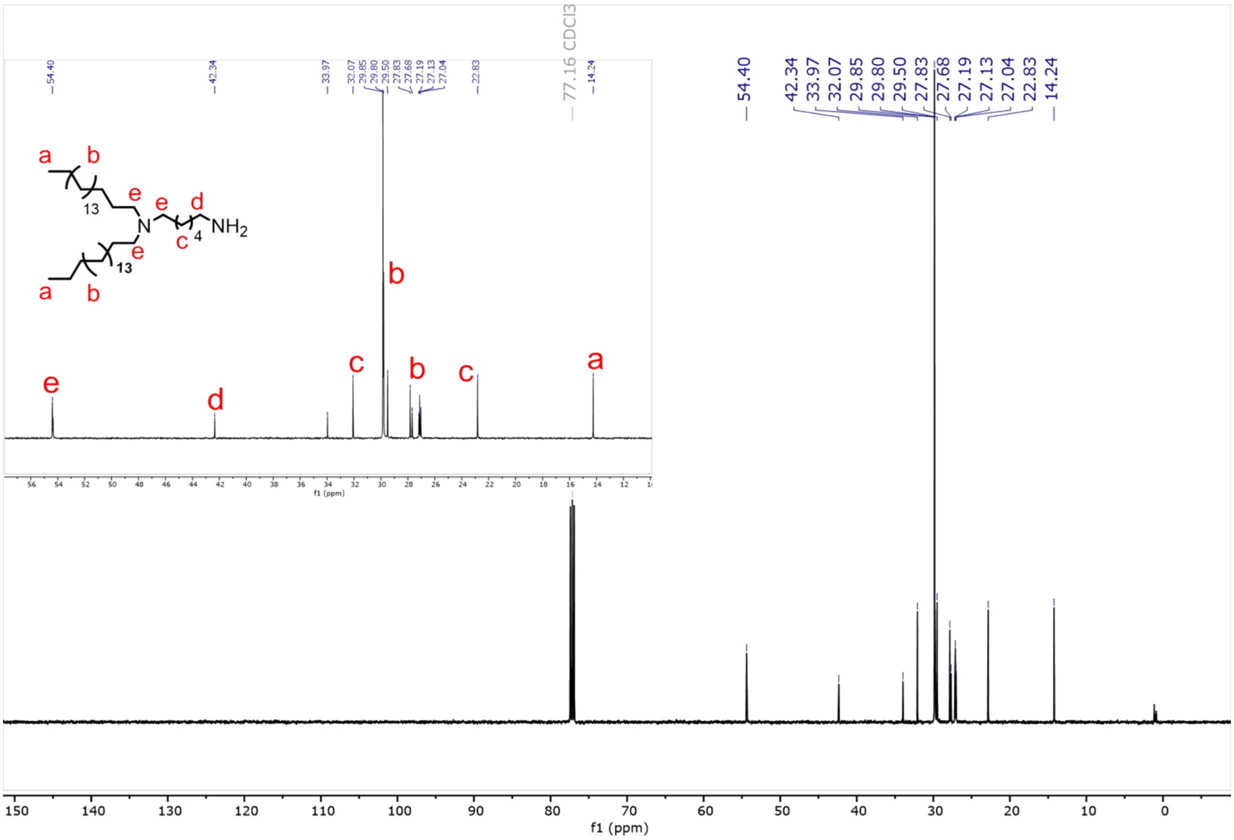


Figure S20: ^{13}C NMR of N-6-aminohexyl-N,N-di-n-octadecylamine

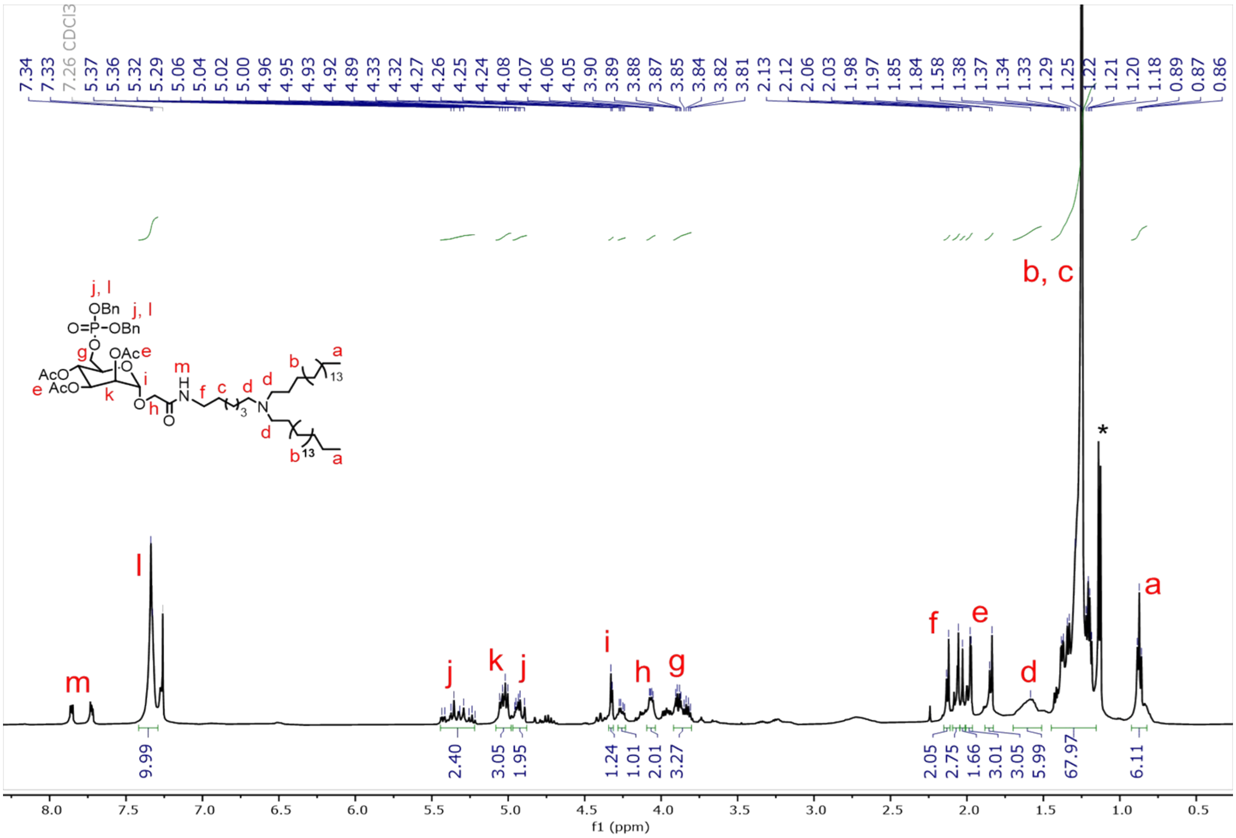


Figure S21: ^{1}H NMR of protected **M6PGL**.

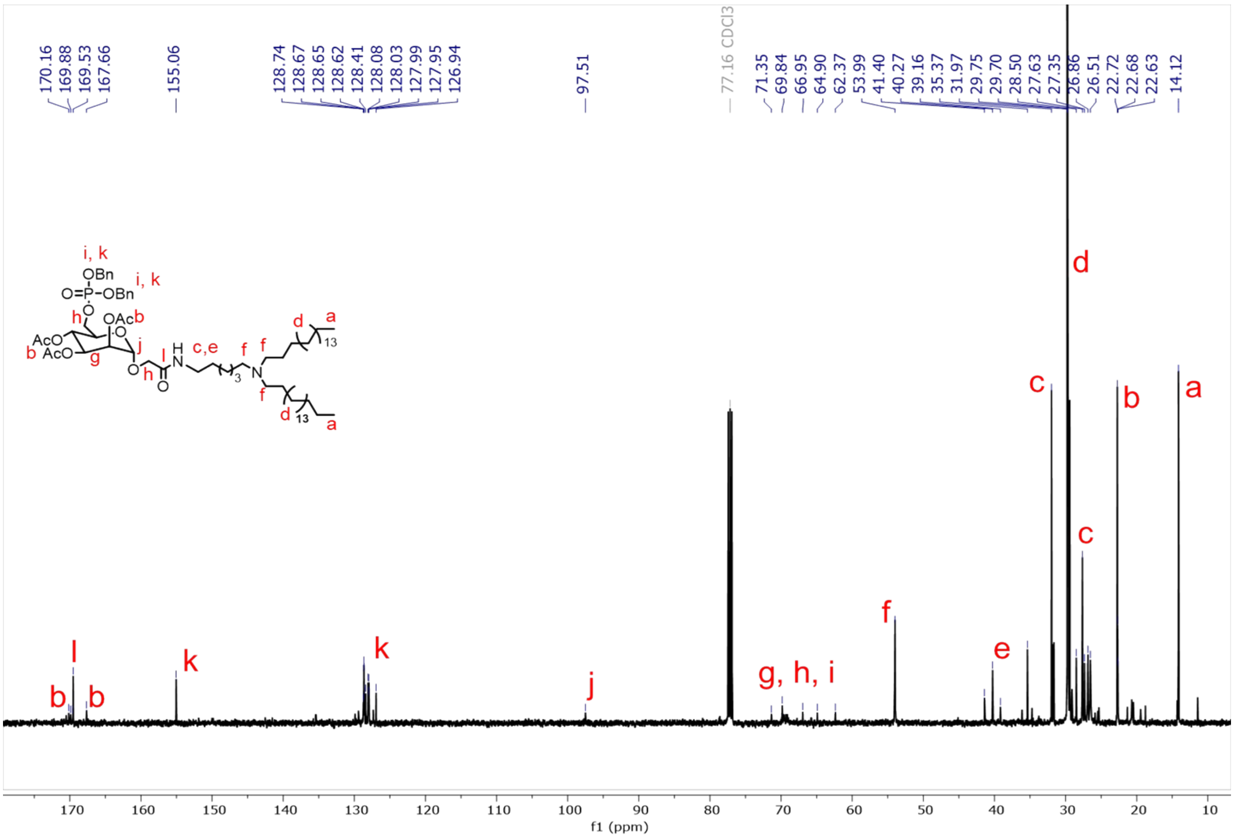


Figure S22: ^{13}C NMR of protected **M6PGL**.

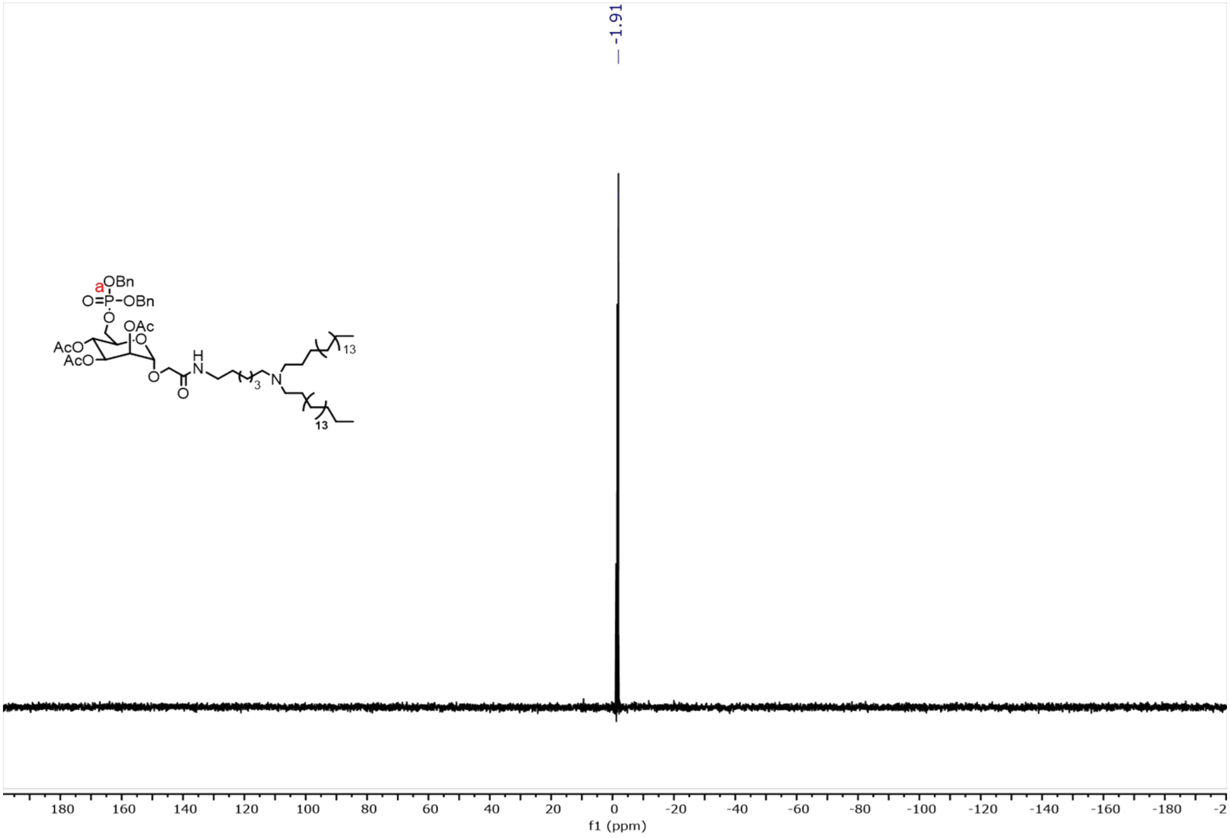


Figure S23: ^{31}P NMR of protected **M6PGL**.

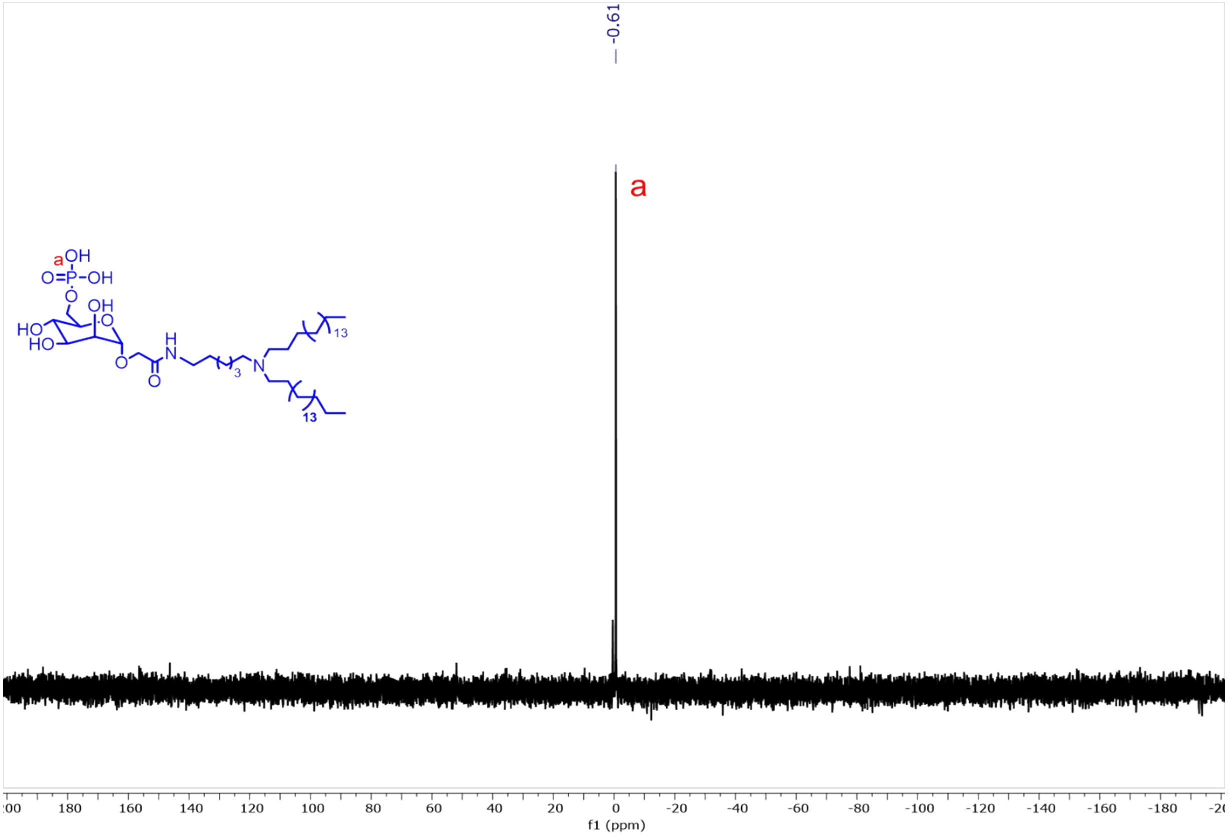


Figure S24: ^{31}P NMR of deprotected **M6PGL**.

References

1. S. Das, N. Parekh, B. Mondal and S. S. Gupta, *ACS Macro Letters*, 2016, **5**, 809-813.
2. B. Mondal, T. Dutta and S. Sen Gupta, *Chem. Commun.*, 2021, **57**, 109-112.