Supporting Information

Design and Synthesis of Shikimoyl-functionalized Cationic Di-block copolypeptide for Cancer Cell Specific Gene Transfection

Abinash Padhy,[a†] Pritam Das,[b,c†] Namita S. Mahadik,[b,c] Sidharth Panda,[a] Mahammad Anas,[d] Sabyasachi Das,[a] Rajkumar Banerjee,*[b,c] Sayam Sen Gupta,*[a]

[a] Department of Chemical Sciences, Indian Institute of Science Education and Research Kolkata, Mohanpur, Nadia, West Bengal 741246, India.

[b] Department of Oils, Lipids Science and Technology, CSIR-Indian Institute of Chemical Technology, Hyderabad 500 007, India.

[c] Academy of Scientific and Innovative Research (AcSIR), CSIR-HRDC Campus, Ghaziabad 201002, India.

[d] School of Chemical Sciences, Indian Association for the Cultivation of Science, Jadavpur, Kolkata 700 032, India.

* Corresponding authors: E-mails- banerjee@iict.res.in (R.B); sayam.sengupta@iiserkol.ac.in (S.S.G), † equal contribution

Materials and methods

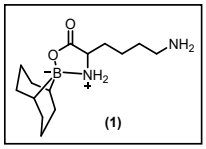
Unless otherwise noted, materials were obtained from commercial suppliers and were used without further purification. Unless otherwise reported, all reactions were performed under argon atmosphere. Removal of solvent in vacuo refers to distillation using a rotary evaporator attached to an efficient vacuum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

All commercially available chemicals used herein were purchased form Merk, India. BF_3Et_2O, Trifluoroacetic acid (TFA) were purchased from SRL chemicals, India. 9-BBN dimer, S-Methylisothiourea hemisulfate salt, N-methyl morpholine, Proton sponge, FL-NHS ester, Agarose, and DNA ladders were purchased from Sigma Aldrich. Shikimic acid, Hexadecylamine, Sodium methoxide were purchased from TCI chemicals, India. L-lysine mono hydrochloride, EDC.HCl, HOBT, N-Ethyldiisopropylamine (DIPEA), Boc anhydride, Triphosgene, and Ethidium bromide were purchased from Spectrochem, India. Dialysis tubing (MWCO=3.5 kDa), DNA loading dye was purchased from ThermoFisher Scientific. Ammnoia soliution, Hydrazine hydrate, 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, for usage in reaction, in a distillation setup. Analytical thin-layer chromatography was performed on pre-coated silica plates obtained from Merk, India. All the column chromatography purification was done with 100-200 mesh silica gel, unless otherwise noted. Solvents were evaporated by a rotary evaporator purchased from Buchi. The eGFP plasmid was received as gift from Prof. Arabinda Chaudhuri's lab at DCS, IISER Kolkata. All polymerization reaction were carried out in Glovebox under argon atmosphere. ^1H and ^{13}C NMR was performed on Bruker NMR Spectrometers (400 or 500 MHz). The NMR spectra was reported relative to the deuterated solvent used. The number average molecular weight (M_n) and dispersity (Đ) were determined by size exclusion chromatography (SEC) using a Waters 1515 isocratic HPLC pump equipped with three Polar Gel-M columns (Mn ~ 0.1-60 kDa (1x); 1-500 kDa (2x)) columns at 45 °C and a Waters 2414 refractive index detector at 35 °C. DMF containing 25 mM LiBr was used as the eluent with a flow rate of 1 mL min^{-1} and narrow PMMA standards were used for calibration. Waters Empower software was used for molecular weight analysis. Agarose gel electrophoresis was performed on Tarsons Midi Submarine Electrophoresis unit with a Tarsons electrophoresis power supply unit. Agarose gels were imaged on a BioRad chemidoc. Dynamic Light scattering measurements and Zeta potential measurements were performed on a Malvern Zetasizer Nano ZS. Transmission electron microscopy (TEM) measurements were performed at 200 kV on a JEOL instrument. Atomic force microscopy (AFM) analysis was performed on an Asylum MFP-3D instrument. Circular Dichroism measurements were performed on JASCO CD spectropolarimeter. For MTT assay, the reading was taken at 570 nm wavelength in a ELISA Synergy H1 Microplate reader instrument. Nikon, Japan confocal microscope was used for all the imaging and BD FACS Canto-II was used for FACS analysis.

Experimental section

Procedure for the synthesis of *N,N'*-di(Boc)-L-homoarginine-NCA monomer

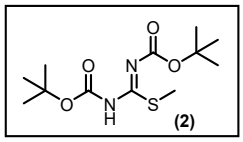
a. Preparation of 9-BBN protected Lysine (1)



L-lysine mono hydrochloride salt was neutralized by treatment with aq. NH_3 solution in a round bottom flask for 30 min at 0 ˚ C and then concentrated under vacuum to remove excess ammonia. This neutralised lysine was directly used for 9-BBN complex formation reaction.

To 150mL of methanol in a 250mL round bottom flask at room temperature under argon was added 0.5 eq. (25 mmol) of 9-BBN dimer. The mixture was heated at reflux until the 9-BBN dimer was completely dissolved (30 min) and to this solution 1 eq. (50 mmol) of neutral lysine was added. The resultant reaction mixture was refluxed for additional 3 h until gas evolution ceased and the suspension became a clear homogenous solution. The methanol was removed on rotary evaporator and the residue was dissolved in hot THF (100 mL), filtered and the filtrate was concentrated to afford a white gummy residue of 9-BBN-L-lysine complex. Excess of 9-BBN was removed by washing with hot hexane and then subjected to high vacuum for 1 h during which time it became an amorphous fluffy solid. This material was used without any further purification for the subsequent coupling reaction.

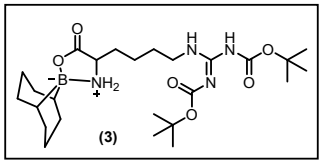
b. Preparation of *N,N'*-di(Boc)-*S*-methylisothiourea (2)



S-Methylisothiourea sulfate (50 mmol, 1 eq) was dissolved in a mixture of H_2O (150 mL) and 1,4-dioxane (150 mL) followed by addition of 1 M aq NaOH solution (100 mL, 100 mmol) and di-tert-butyl dicarbonate (Boc_2O) (250 mmol, 5 eq). The reaction mixture was vigorously stirred for 24 h at r.t. The precipitate formed was filtered and washed with a small amount of H_2O. The filtrate was concentrated under reduced pressure to approximately half the volume and the solid was separated by filtration. The solids were combined and suspended in H_2O (1000 mL) at approximately 50 °C for 12 h, and filtered. It was then worked up with DCM & H_2O (thrice) and finally was then dried over P_2O_5 to get **2**. The yield was found to be 95% (47.5 mmol).

^{1}H NMR (500 MHz, $CDCl_3$) δ 1.50 (s, 18H), 2.38 (s, 3H), 11.59 (s, 1H). **^{13}C NMR (126 MHz, $CDCl_3$)** δ 14.49, 28.15, 81.04, 83.32, 150.86, 160.85, 171.51. **HRMS (ESI-TOF):** m/z $[M+Na]^+$: Calculated-313.1198, Experimental-313.1208.

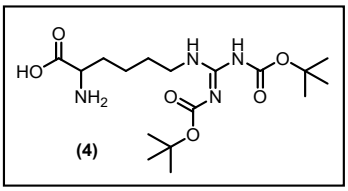
c. Preparation of *N,N'*-di(Boc)-L-homoarginine 9-BBN complex (3)



In a 100 ml schlenk tube, 9-BBN lysine complex 1 eq. (5 mmol) was taken, and applied to high vacuum for 15 mins. Dry THF (~20 ml) was added to the tube under argon atmosphere followed by addition of 1 eq. of DIPEA at 0 ˚C. The solution was stirred for 5 mins and then 1.2 eq. (6 mmol) of *N,N'*-di(Boc)-S-methylisothiourea was added under stirring. The reaction was allowed to stir at 40 ˚C for 16 hours. After completion of reaction (checked by TLC), the solvent was evaporated in vacuuo and the pure product was isolated by column chromatography with EtOAc and Hexane (4:6 v/v). The yield of the reaction is found to be around 75% (3.75 mmol).

^{1}H NMR (500 MHz, $CDCl_3$) δ 0.52 (s, 2H), 1.45 (s, 20H), 1.49 – 1.66 (m, 8H), 1.72 - 1.83 (m, 8H), 3.31 (q, 2H), 3.70 – 3.74 (m, 1H), 4.62 – 4.66 (m, 1H), 5.72 – 5.77 (m, 1H), 8.35 (s, 1H). **^{13}C NMR (126 MHz, $CDCl_3$)** δ 23.20, 23.93, 24.44, 28.08, 28.35, 28.54, 30.76, 31.22, 31.39, 31.74, 40.51, 55.30, 79.63, 83.42, 153.28, 156.33, 163.25, 174.21. **HRMS (ESI-TOF):** m/z $[M+H]^+$: Calculated- 509.3510, Experimental- 509.3469.

d. Preparation of *N,N'*-di(Boc)-L-homoarginine (4)

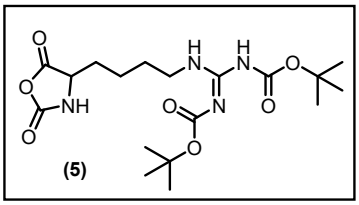


(4)

The cleavage of 9-BBN complex was done by treating compound **3** with MeOH:$CHCl_3$ (in 1:5 ratio), and then it was stirred at 40 ˚C for 24 hours. After complete cleavage of 9-BBN complex (from TLC), the reaction mixture was concentrated and precipitated with hot hexane to remove excess 9-BBN. After three to four rounds of precipitation, the product was isolated with maximum purity. (Yield ~95%)

^{1}H NMR (400 MHz, CHLOROFORM-D) δ 1.45-1.59 (s, 22H), 1.86-1.93 (t, 2H), 3.32 – 3.35 (t, 2H), 3.75 (s, 1H), 7.98 (bs, 2H), 8.35 (s, 1H), 11.46 (s, 1H). **^{13}C NMR (101 MHz, CHLOROFORM-D)** δ 23.01, 28.14, 28.38, 28.82, 29.76, 30.74, 40.87, 54.59, 79.50, 83.12, 153.15, 156.16, 163.24, 174.19. **HRMS (ESI-TOF):** m/z $[M+H]^+$: Calculated- 389.2400, Experimental- 389.2401.

e. Preparation of *N,N'*-di(Boc)-L-homoarginine-NCA (5)



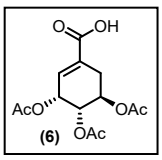
(5)

To a solution of *N,N'*-di(Boc)-L-Homoarginine (2 mmol) in freshly distilled anhydrous tetrahydrofuran (10 mL) was added N-methylmorpholine (2 mmol) and stirred for 5 mins at room temperature under argon atmosphere. A solution of triphosgene (1 mmol) in anhydrous tetrahydrofuran (5 mL) under argon atmosphere was added and the reaction mixture was heated to 60 ˚ C for 1.5 h. After completion of reaction (via TLC and FT-IR), the reaction mixture was cooled to room temperature following which the solids were removed using a glass fritted funnel. The clear solution was added to dry hexane with stirring in a beaker under ice bath and the formation of an off-white precipitate was observed. The solids were vacuum filtered using another glass frit funnel and re-dissolved in dry ethyl acetate and then purification was done using anhydrous flash column chromatography. The column was packed with vacuum oven dried silica (60-120 mesh) in dry hexane. The crude product was purified with a gradient of freshly distilled 1:4 EtOAc/ hexane through the column and then 100% dry EtOAc as the eluent. The fraction containing desired product was dried under vacuum to yield a white fluffy solid NCA (Yield 1.4 mmol, 70%). The pure isolated NCA was taken inside the glovebox for recrystallization with THF/pentane mixture and then proceeded for polymerization.

^{1}H NMR (400 MHz, CHLOROFORM-D) δ 1.43 (s, 20H), 1.57 – 1.63 (m, 2H), 1.78 – 1.82 (m, 1H), 1.93 – 1.98 (m, 1H), 3.32 – 3.4 (m, 2H), 4.31 – 4.34 (t, 1H), 7.73 (s, 1H), 8.45 (t, 1H), 11.38 (s, 1H). **^{13}C NMR (101 MHz, CHLOROFORM-D)** δ 20.20, 21.58, 28.00, 28.17, 28.26, 30.78, 40.06, 57.45, 80.18, 83.64, 152.31, 153.12, 156.18, 162.48, 170.36. **FT-IR:** 1847, 1776 cm^{-1}.

Procedure for the synthesis of 3,4,5-tri-acetyl shikimic-L-lysine-NCA monomer

a. Preparation of 3,4,5-tri-acetyl shikimic acid (6)

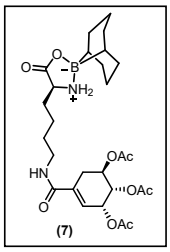


(6)

To a suspension of shikimic acid (5 g, 16.65 mmol) in dry pyridine (25 mL) 6 eq. of acetic anhydride (9.5 mL, 100 mmol) was added dropwise at 0 ˚C and the reaction mixture was stirred at room temperature for 12 h. After completion of the reaction (via TLC), pyridine solvent was evaporated in vacuo. Then the crude product was dissolved in DCM and washed with 2N HCl to quench pyridine. The DCM solution was washed with water and brine followed by drying over sodium sulfate. The DCM part was removed by rotary evaporation to obtain completely pure 3,4,5-tri-acetyl shikimic acid (Yield ~99%).

^{1}H NMR (500 MHz, $CDCl_3$) δ 1.98 – 2.06 (m, 9H), 2.38 (dd, 1H), 2.84 (dd, 1H), 5.19 – 5.27 (m, 2H), 5.70 (s, 1H), 6.81 (s, 1H), 9.70 (s, 1H). **^{13}C NMR (126 MHz, $CDCl_3$)** δ 20.70, 20.71, 20.95, 28.05, 66.04, 66.78, 67.53, 130.69, 134.91, 170.02, 170.09, 170.16, 177.35.

b. Preparation of 3,4,5-tri-acetyl shikimic acid 9-BBN-L-lysine complex (7)

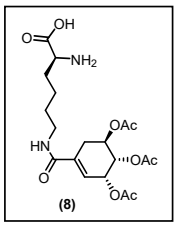


(7)

To a solution of tri-acetyl shikimic acid (10 mmol) in dry THF (50 mL) was added N-(3-Dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (12 mmol), Hydroxybenzotriazole (12 mmol) and DMAP (1 mmol) under ice bath successively. After 10 mins of stirring 9- BBN-L-lysine complex (compound **1**) (9.8 mmol) in dry THF (10 ml) was added to the reaction mixture and was stirred for 12 hours. After completion of the reaction (via TLC), the solvent in reaction mixture was removed by rotary evaporation. The crude mixture was dissolved in EtOAc (100 ml) and washed successively with 10% citric acid solution (2 x 100 ml), aq. sodium bicarbonate solution (2 x 100 ml), brine solution (100 mL). Finally, the organic layer was dried over anhydrous sodium sulfate and concentrated by rotary evaporation. The desired coupling product was obtained by column purification using hexane/EtOAc (1:4) as eluent (yield 8.5 mmol, 85 %).

^{1}H NMR (500 MHz, $CDCl_3$) δ 0.52 (s, 2H), 1.44 (s, 4H), 1.51 – 1.66 (m, 8H), 1.71 – 1.78 (m, 4H), 1.79 – 1.85 (m, 2H), 2.01 – 2.06 (m, 9H), 2.35 (dd, 1H), 2.95 (dd, 1H), 3.24 – 3.30 (t, 2H), 3.74 (s, 1H), 4.87 (t, 1H), 5.16 (dd, 1H), 5.21 – 5.27 (m, 1H), 5.56 (t, 1H), 5.64 (t, 1H), 6.36 (dd, 1H), 6.83 (t, 1H). **^{13}C NMR (126 MHz, $CDCl_3$)** δ 20.82, 20.88, 21.06, 22.41, 22.43, 24.00, 24.46, 29.18, 29.41, 29.77, 30.00, 31.29, 31.42, 31.48, 31.76, 38.80, 55.31, 66.16, 66.88, 68.60, 126.61, 135.45, 167.26, 170.20, 170.33, 170.36, 174.68.

c. Preparation of 3,4,5-tri-acetyl shikimic-L-lysine complex (8)

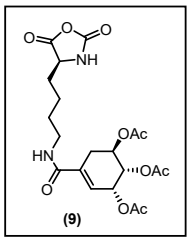


(8)

The cleavage of 9-BBN complex was done by treating compound **7** with MeOH:$CHCl_3$ (in 1:5 ratio), and then it was stirred at 40 ˚C for 24 hours. After complete cleavage of 9-BBN complex (from TLC), the reaction mixture was concentrated and precipitated with hot hexane to remove excess 9-BBN. After two to three rounds of precipitation, the product was isolated with maximum purity. (Yield ~95%)

^{1}H NMR (500 MHz, $CDCl_3$) δ 1.54 (s, 4H), 1.87 – 2.15 (m, 9H), 2.38 (d, 1H), 2.70 (d, 1H), 3.01 (d, 1H), 3.24 (s, 2H), 3.61 – 3.82 (m, 1H), 3.95 (s, 1H), 5.10 – 5.35 (m, 2H), 5.66 (s, 1H), 6.44 (s, 1H), 7.48 (s, 1H), 8.04 (s, 2H). **^{13}C NMR (126 MHz, $CDCl_3$)** δ 20.85, 20.92, 21.10, 22.67, 28.88, 29.65, 30.37, 39.77, 54.33, 66.21, 66.95, 69.12, 126.50, 135.72, 167.03, 170.32, 170.47, 173.84.

d. Preparation of 3,4,5-tri-acetyl shikimic-L-lysine-NCA monomer (9)



(9)

To a solution of 3,4,5-tri-acetyl shikimic-L-lysine complex (2 mmol) in freshly distilled anhydrous tetrahydrofuran (10 mL) was added 1 eq. (2 mmol) of N-methylmorpholine and stirred for 5 mins at room temperature. A solution of triphosgene (1 mmol) in anhydrous tetrahydrofuran (5 mL) under argon atmosphere was then added and the reaction mixture was heated to 60 ˚ C for 1.5 h. After completion of reaction (via TLC and FT-IR), the reaction mixture was cooled to room temperature following which the solids were removed using a glass fritted funnel. The clear solution was added to dry hexane with stirring and the formation of a white precipitate was observed. The white solids were vacuum filtered using another glass frit funnel and re-dissolved in dry ethyl acetate and further purification was done using anhydrous flash column chromatography. The column was packed with vacuum oven dried silica (60-120 mesh) in dry hexane. The crude product was purified with a gradient of freshly distilled 1:3 EtOAc/ hexane and 100% EtOAc through the column as the eluent. The fraction containing desired product was dried under vacuum to yield a white fluffy solid NCA (Yield 1.8 mmol, 90%). The pure isolated NCA was taken inside the glovebox for recrystallization with THF/hexane mixture and then proceeded for polymerization.

^{1}H NMR (400 MHz, CHLOROFORM-D) δ 1.55 (s, 4H), 1.81 (s, 1H), 1.88 – 2.21 (m, 11H), 2.32 (d, 1H), 2.98 (s, 2H), 3.39 (d, 2H), 3.73 (d, 1H), 4.33 (s, 1H), 5.20 (d, 2H), 5.64 (s, 1H), 6.37 (s, 1H), 6.72 (s, 1H), 8.14 (s, 1H). **^{13}C NMR (126 MHz, $CDCl_3$)** δ 20.63, 20.70, 20.87, 21.89, 28.37, 29.27, 31.06, 31.42, 36.53, 57.44, 66.00, 66.68, 68.55, 125.94, 135.57, 152.32, 162.70, 166.63, 169.97, 170.11, 170.63. **FT-IR:** 1849, 1778 cm^{-1}.

Sample preparation for SEC analysis

The protected di-block copolypeptide (**HDA-*b*-PHAG$_m$-*b*-PLSA$_n$)** and homo-polypeptide (**HDA-PHAG$_m$)** samples were prepared at concentrations of 5-10 mg mL^{-1}. All the samples were filtered using 0.22 µm syringe filter. Prior to sample injection system was calibrated by poly-methylmethacrylate (PMMA) standards. A constant flow rate of 1 mL min^{-1} was maintained.

Circular Dichroism measurement

All the polypeptides were dissolved in nuclease free water and filtered through 0.22 µm syringe filters to remove dust particles. The concentration of polypeptides was adjusted to 1.0–0.25 mg mL^{-1}, and CD (190–350 nm) spectra of the polypeptides were recorded using JASCO J-815 CD spectro-photometer in a cuvette with 1 mm path length. All the spectra were recorded for an average of three scans, and the spectra were reported as a function of molar ellipticity [θ] versus wavelength.

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 one averaged for 5 scans and averaged over to present the data. DLS of all the block-copolypeptides were measured in water at 0.125 mg/ml concentration after filtering it through 0.22µm nylon syringe filters. DLS of the polyplex solution was measured at different charge ratios.

Sample preparation for TEM and Cryo-TEM study

Firstly, the polyplex was formulated at N/P 10:1 ratio following the method provided in Experimental section and a solution (5 µL) was drop-casted onto a 200-mesh copper carbon-coated grid and maintained for full adsorption. The grids were stained with 2 µL of uranyl acetate solution (0.2 mg mL^{-1}) for 10 seconds and washed with DI water thrice. For cryo analysis, the polyplex sample was drop-casted onto a 200-mesh copper carbon-coated grid and 2 µL of uranyl acetate stain solution was given to it and dipped into liquid ethane to fix the polyplexes at native hydrated state. Images were recorded using a Joel-1200 TEM instrument.

Sample preparation for AFM study

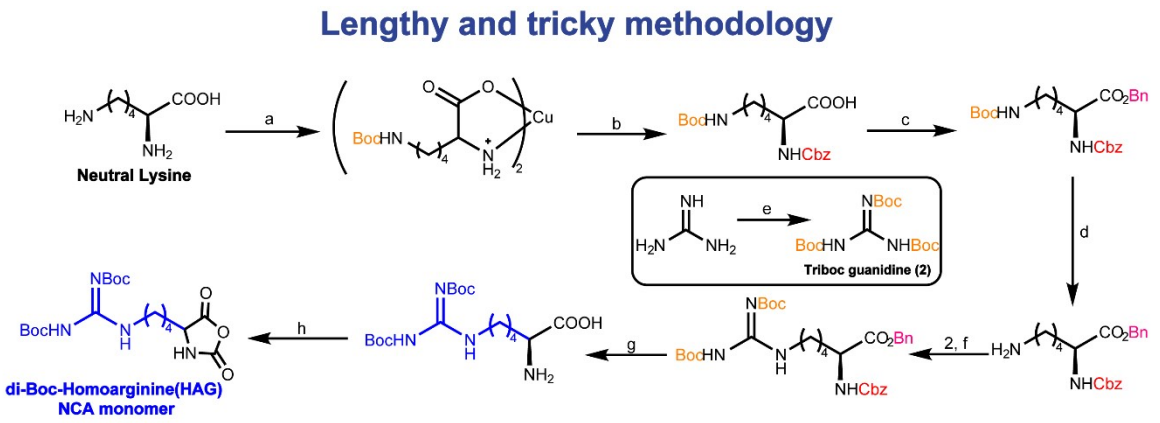
Firstly, the polyplex was formulated at N/P 10:1 ratio following the method provided in Experimental section and a solution (5 µL) was drop-casted onto a piece of silicon wafer and maintained for full adsorption. Images were recorded using an Asylum MFP-3D AFM instrument.

Cellular internalization study before and after mannan treatment using confocal microscopy

HEK 293T, MDA-MB-231, and RAW 264.7 cells were seeded in a 12-well plate at a density of 5 × 10^4 cells per well in DMEM containing 10% FBS. The plate was incubated at 37 °C with 5% CO_2 for 18-24 h. After that, the incubation medium was replaced with 300 ug mannan for 30 mins. After that, polypeptide treatment was done and incubated for 4 h at 37° C with 5% CO_2. Lysosomes were stained with 50 nM LysoTracker Red-DND (50 nM) in DMEM, and cells were incubated for 30 min at 37 °C with 5% CO_2. After 30 min of incubation, cells were incubated with Hoechst 33342 (80 µM) for 10 minutes to stain the nuclei. Images were acquired in Nikon, Japan confocal platform by using dry immersion 20× objective and were analysed using the NIS Elements analysis (version 4.50) software.

Cellular transfection study by FACS

After confocal imaging, all the cells were washed once with 1X PBS solution and then trypsinized and again resuspended in 500 µl 1X PBS. The cells were then analysed by FACS (BD FACS Canto II Software).



a) $CuSO_4$. $5H_2O$, 2M $NaHCO_3$, Boc_2O, 12h, RT; b) (i) 1,8-quinolinol, 16h, RT; (ii) CbzCl, 12h, RT; c) BnBr, NaH, DMF, 16h, RT; d) THF, 1N HCl, 6h, RT; e) Boc_2O, Na_2CO_3, DMSO, 72h, 40 °C; f) TEA, dry THF, 24h, RT; g) H_2/Pd, dry MeOH, 12 h, RT; h) Triphosgene, N-methyl morpholine, dry THF, 1.5h, 60 °C

Fig. S1: Previous reported methodology for the synthesis of **HAG**-NCA monomer.[1]

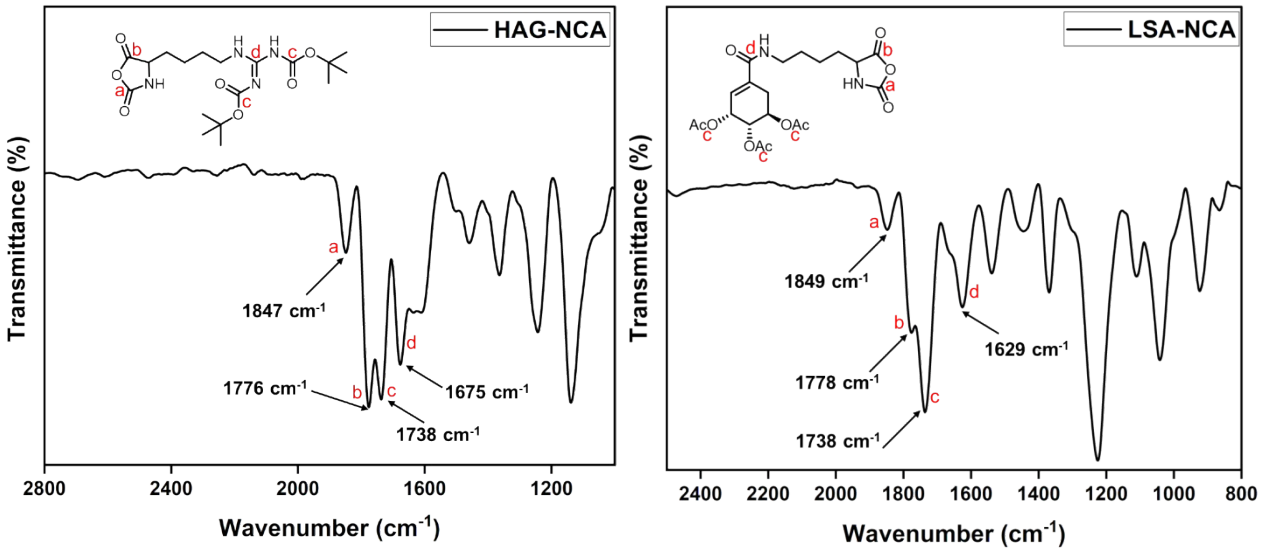
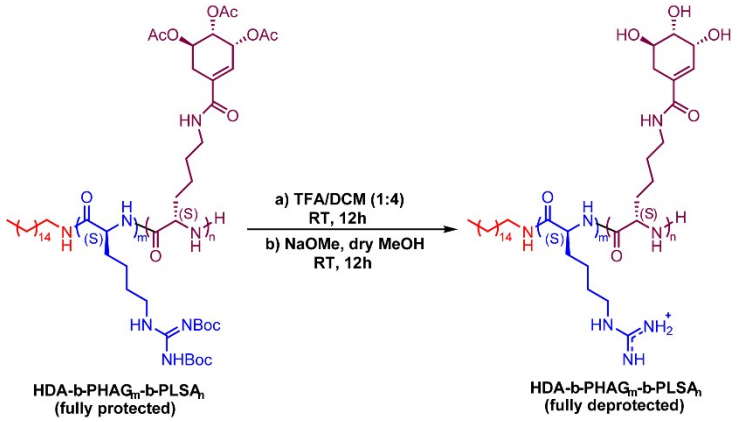


Fig. S2: FT-IR spectra of HAG-NCA and LSA-NCA representing two unsymmetrical anhydride stretches (1847/1849 cm^{-1} and 1776/1778 cm^{-1}) of NCA ring with other type of C=O present in the compound.



Scheme S1: Deprotection of Boc- and acetyl group of HAG and LSA block respectively.

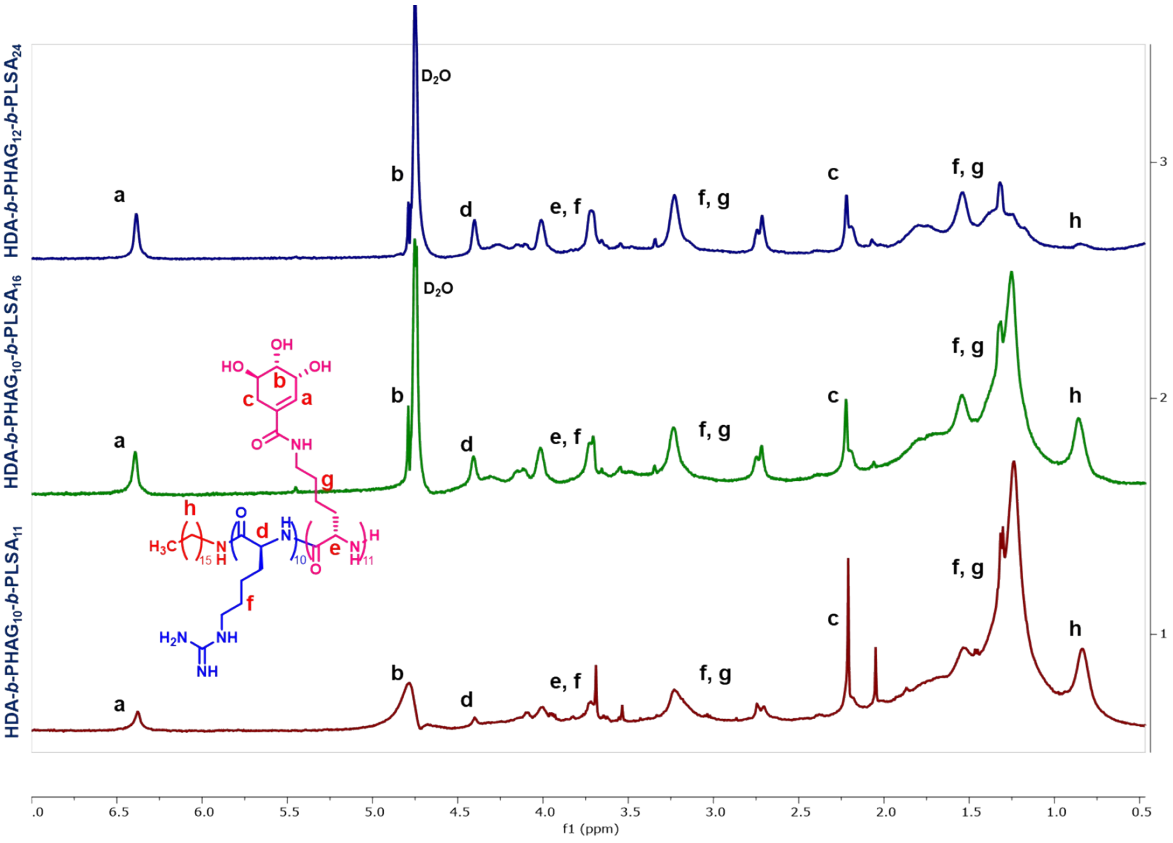


Fig. S3: ^{1}H NMR spectra of fully deprotected di-block polypeptides.

Discussion: The absence of proton peaks at 1.47 and 2.04 ppm indicated the deprotection of the Boc- group from the HAG block, and the acetyl group from the LSA block, respectively.

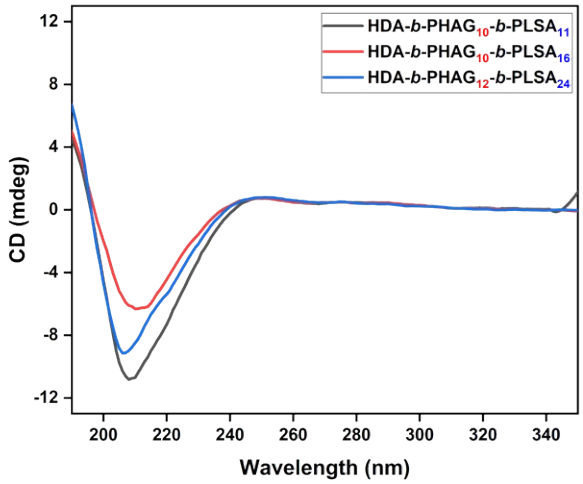


Fig. S4: Circular dichroism spectra of deprotected polypeptides in PBS 7.4 showing random coil confirmation.

Discussion: The peak at 205-210 nm in the CD spectra indicated the random coil conformation of the di-block copolypeptides.

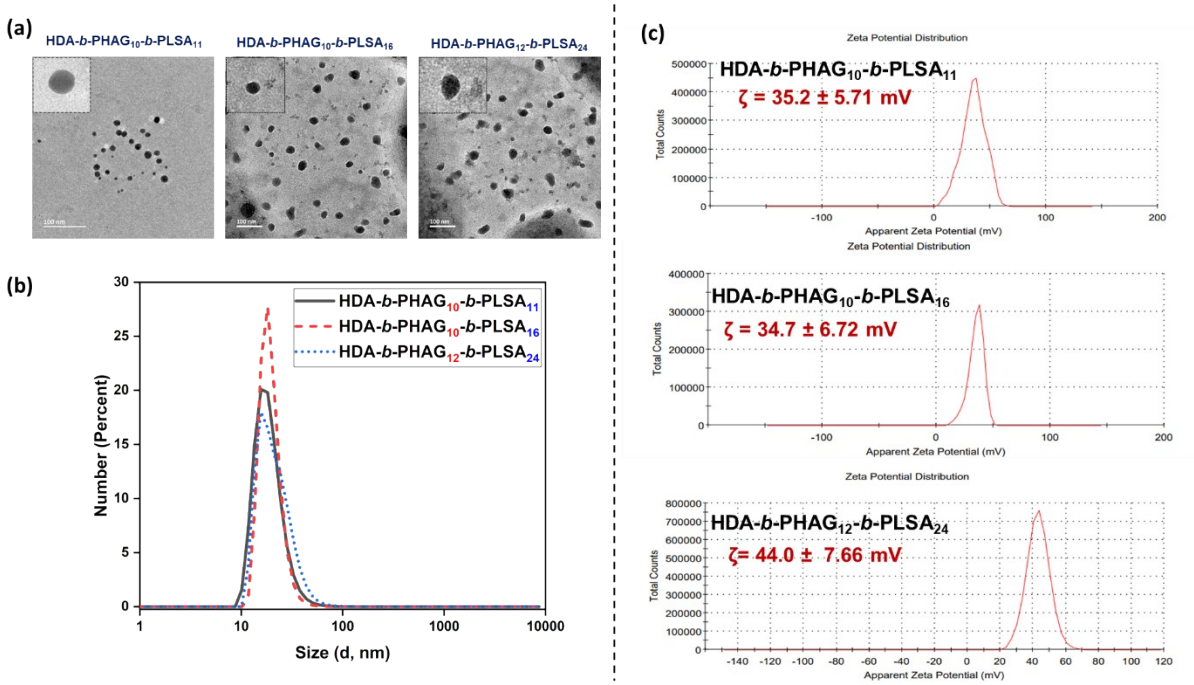


Fig. S5: Characterization of fully deprotected di-block copolypeptides; (a) TEM showing spherical morphology, (b) DLS analysis showing aggregates of ~20 nm diameter, (c) zeta potential analysis showing positive surface charge of the cationic di-block copolypeptides.

Table 1: Size and charge details of di-block copolypeptides from DLS and zeta potential measurement.

S.N	Polypeptide	Diameter (D_h, nm)	PDI	Zeta Potential (ζ)
1	HDA-*b*-$PHAG_{10}$-*b*-$PLSA_{11}$	19.2 ± 6.9	0.42	35.2 ± 5.7
2	HDA-*b*-$PHAG_{10}$-*b*-$PLSA_{16}$	19.6 ± 5.3	0.62	34.7 ± 6.7
3	HDA-*b*-$PHAG_{12}$-*b*-$PLSA_{24}$	21.8 ± 9.1	0.52	44.0 ± 7.7

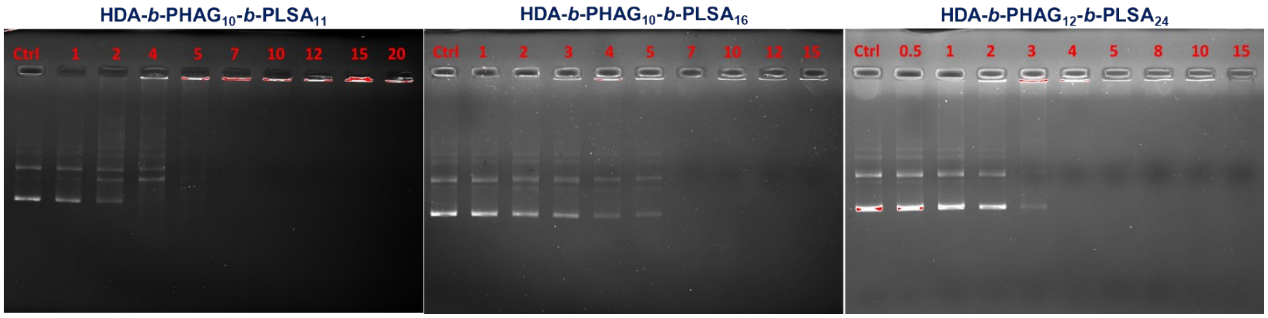


Fig. S6: DNA complexation studies using Agarose gel electrophoretic mobility shift assay showing at which N/P ratio (red colour numbers on top of each image) full complexation of DNA with cationic di-block copolypeptide is happening.

Discussion: For HDA-*b*-$PHAG_{10}$-*b*-$PLSA_{11}$ and HDA-*b*-$PHAG_{10}$-*b*-$PLSA_{16}$, complexation starts at 4:1 and full complexation occurs at 7:1. For HDA-*b*-$PHAG_{12}$-*b*-$PLSA_{24}$, complexation starts at 2:1 and full complexation occurs at 4:1.

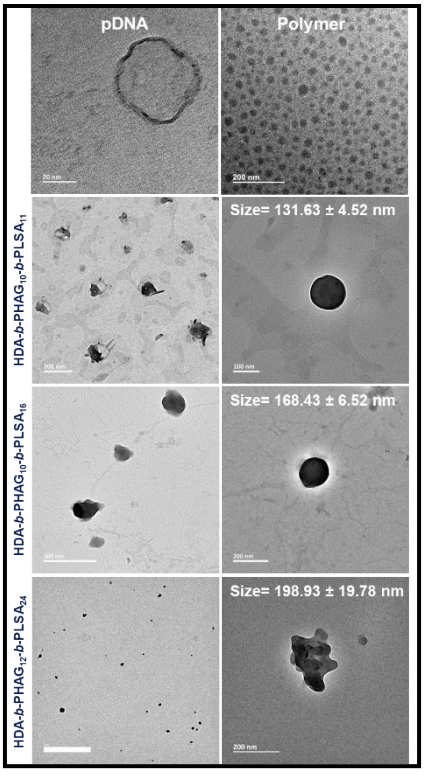


Fig. S7: Dried state TEM images of plasmid DNA, polypeptide, and polyplexes showing spherical morphology.

Discussion: Dried state TEM images of polyplexes showed distorted spherical morphology and sizes vary from ~130 to 200 nm.

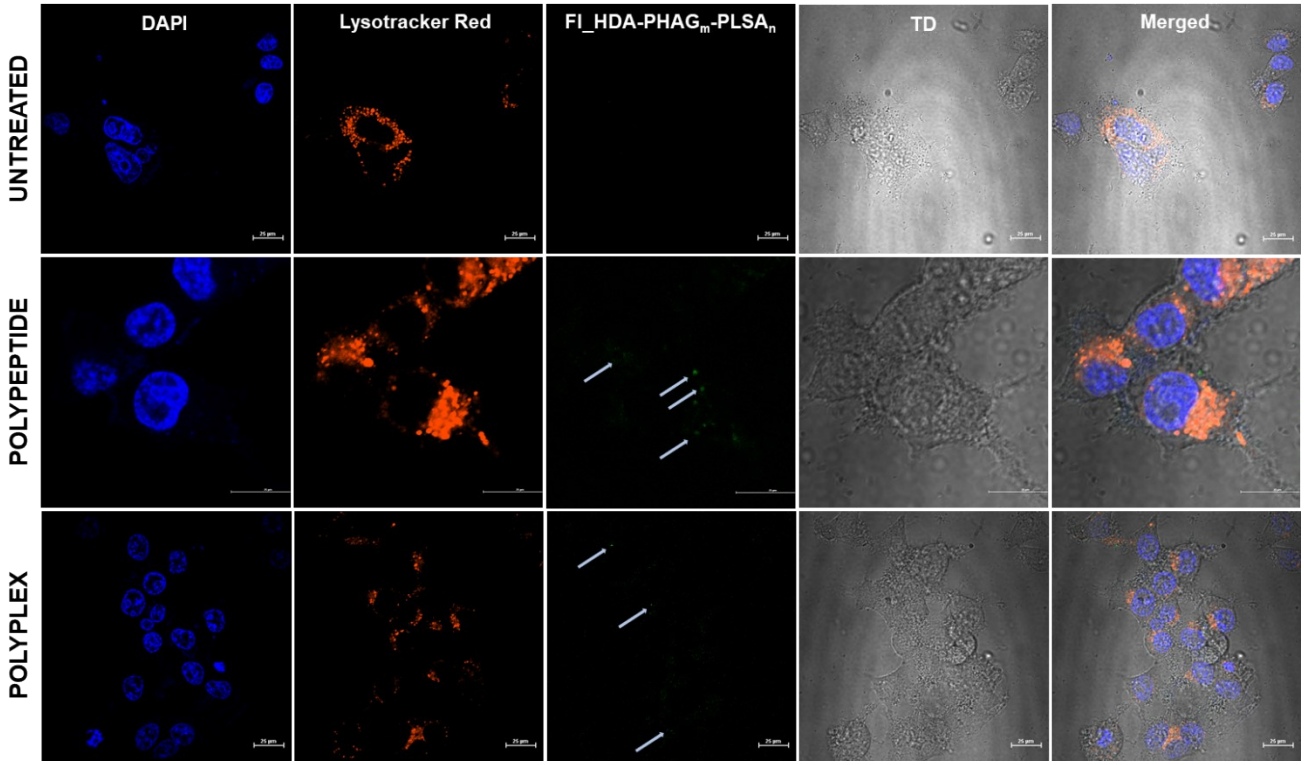


Fig. S8: Cellular uptake experiment of fluorescein tagged HDA-*b*-PHAG$_m$-*b*-PLSA$_n$ on HEK 293T cells for 4 h. HEK 293T cells were cultured for 4 h with **Fl_HDA-*b*-PHAG$_m$-*b*-PLSA$_n$** (12 µg mL^{-1}) in DMEM and then stained with LysoTracker Red (50 nM) (for staining lysosomes) for 30 min and Hoechst 33342 (80 µM) (for staining nuclei) for 10 min. The cells were probed by confocal microscopy. (scale bars, 25 µm).

Discussion: The green punctate like spots evidenced for the entry of the polypeptides and polyplexes which was mostly seen in the cytoplasmic region. The polyplex internalization is less in comparison to the polypeptides which can be attributed due to their larger size and less cationic charge.

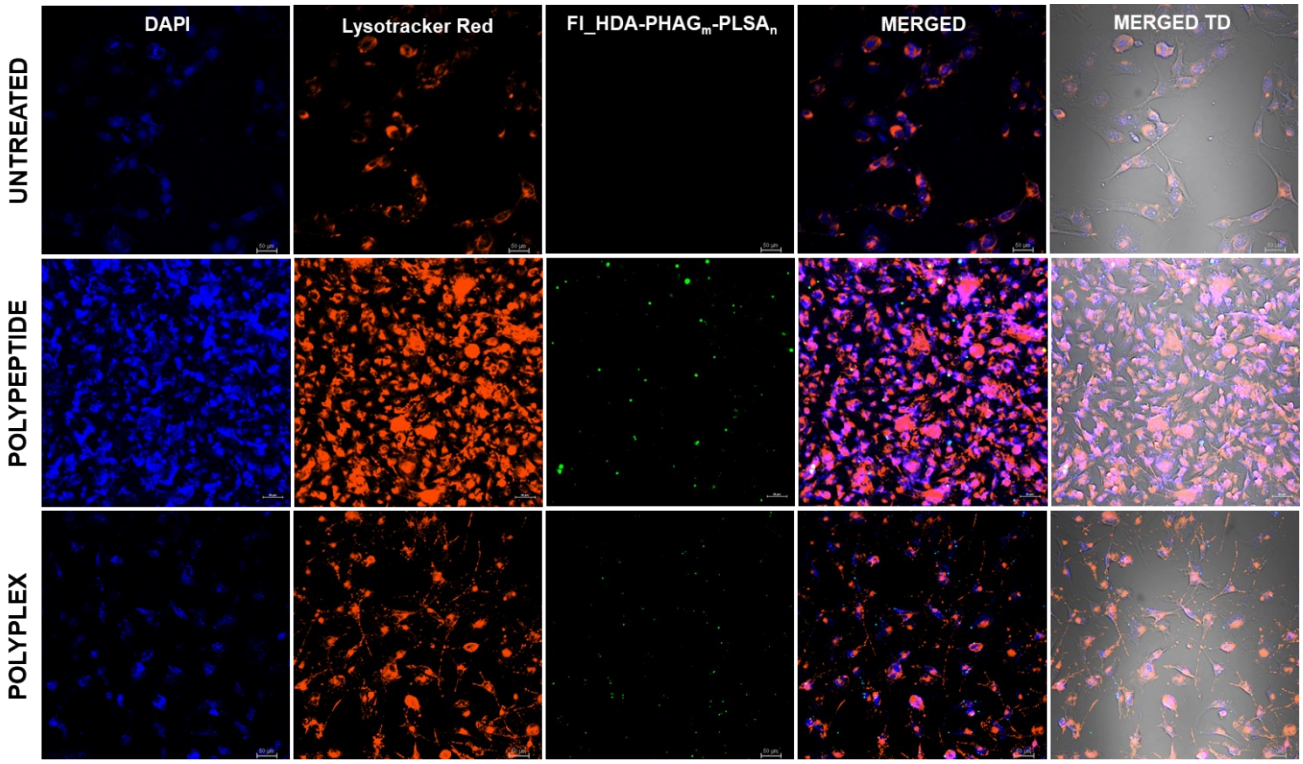


Fig. S9: Cellular uptake experiment of fluorescein tagged HDA-*b*-PHAG$_m$-*b*-PLSA$_n$ on MDA-MB-231 cells for 4 h. MDA-MB-231 cells were cultured for 4 h with **Fl_HDA-*b*-PHAG$_m$-*b*-PLSA$_n$** (12 µg mL^{-1}) in DMEM and then stained with LysoTracker Red (50 nM) (for staining lysosomes) for 30 min and Hoechst 33342 (80 µM) (for staining nuclei) for 10 min. The cells were probed by confocal microscopy. (scale bars, 50 µm).

Discussion: The green punctate like spots evidenced for the entry of the polypeptides and polyplexes which was mostly seen in the cytoplasmic region. The polyplex internalization is less in comparison to the polypeptides which can be attributed due to their larger size and less cationic charge.

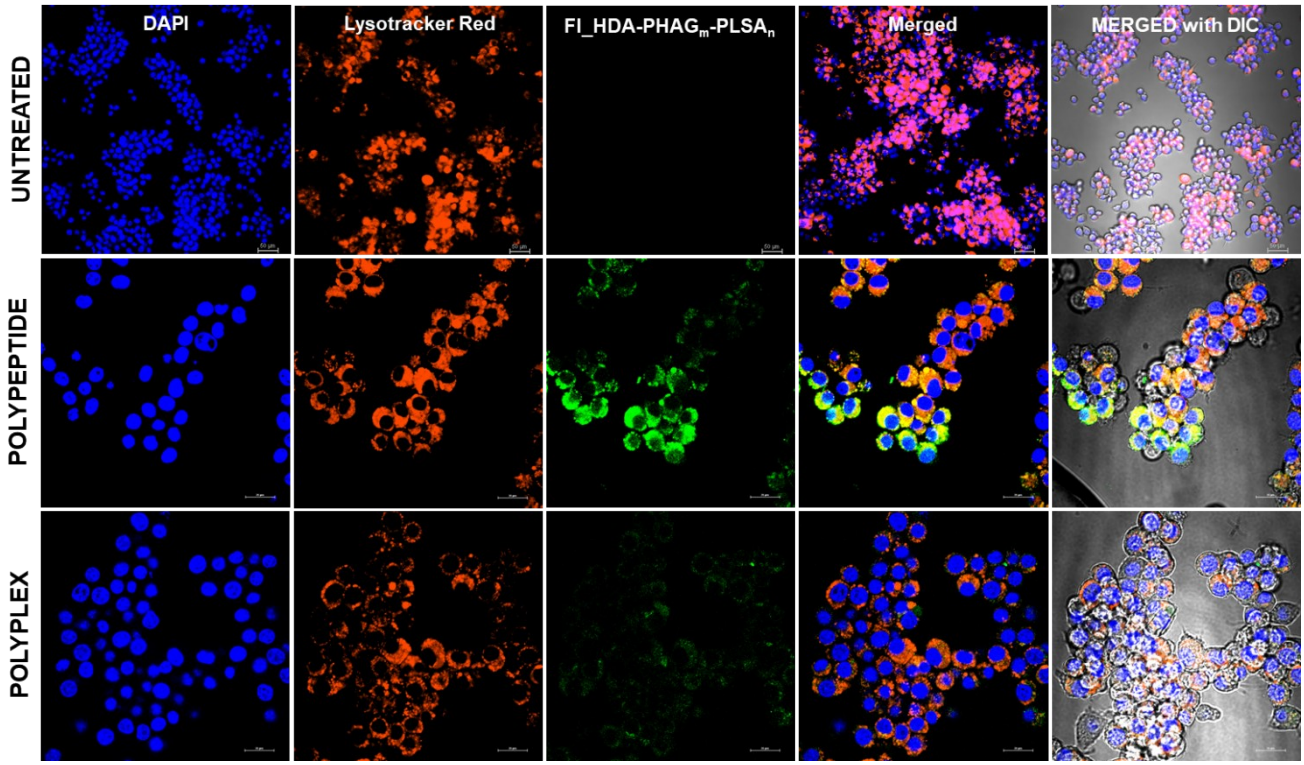


Fig. S10: Cellular uptake experiment of fluorescein tagged HDA-*b*-PHAG$_m$-*b*-PLSA$_n$ on RAW 264.7 cells for 4 h. RAW 264.7 cells were cultured for 4 h with **Fl_HDA-*b*-PHAG$_m$-*b*-PLSA$_n$** (12 µg mL^{-1}) in DMEM and then stained with LysoTracker Red (50 nM) (for staining lysosomes) for 30 min and Hoechst 33342 (80 µM) (for staining nuclei) for 10 min. The cells were probed by confocal microscopy. (scale bars, 25 µm).

Discussion: The green punctate like spots evidenced for the entry of the polypeptides and polyplexes which was mostly seen in the cytoplasmic region. The polyplex internalization is less in comparison to the polypeptides which can be attributed due to their larger size and less cationic charge.

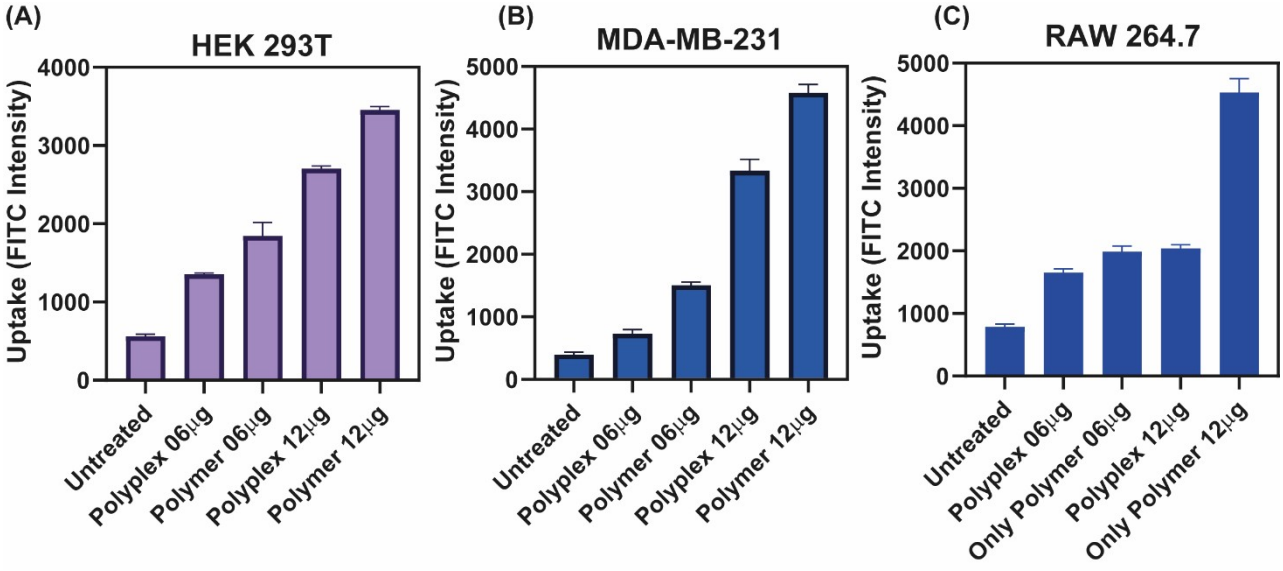


Figure S11: Flow cytometry assay for the cellular uptake of fluorescein tagged HDA-PHAG$_m$-PLSA$_n$ (only polymer) and polyplex (complex of plasmid DNA & polymer) on HEK 293T, MDA-MB-231, and RAW 264.7 cell lines for 4 h. HEK 293T (A), MDA-MB-231 (B), and RAW 264.7 (C) cells were cultured for 4 h with **Fl_HDA-PHAG$_m$-PLSA$_n$** (6/12 µg mL^{-1}) & polyplex (6/12 µg mL^{-1}) in DMEM.

Discussion: Larger size and reduced cationic charge of polyplexes than corresponding polypeptides aided slightly less internalization.

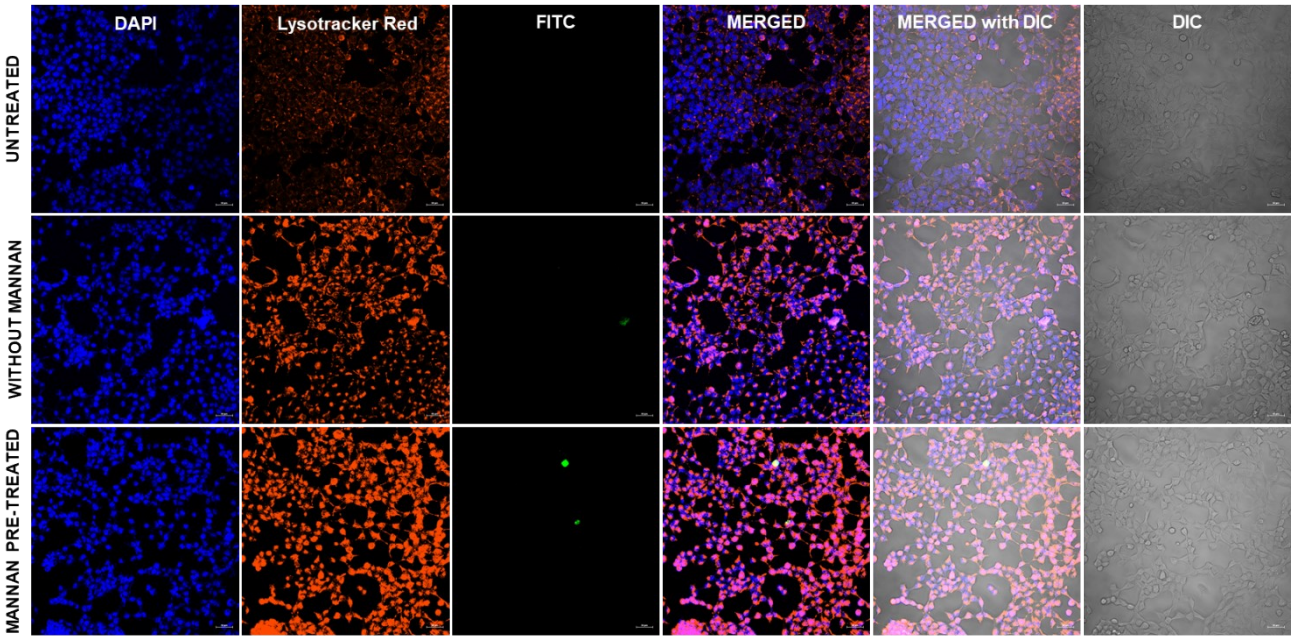


Figure S12: Cellular uptake experiment of fluorescein tagged **HDA-*b*-PHAG$_m$** on HEK 293T cells for 4 h. HEK 293T cells were cultured for 30 mins with mannan (300 µg) and then cultured for 4 h with **Fl_HDA-*b*-PHAG$_m$** (50 µg mL^{-1}) in DMEM and then stained with LysoTracker Red (50 nM) (for staining lysosomes) for 30 min and Hoechst 33342 (80 µM) (for staining nuclei) for 10 min. The cells were probed by confocal microscopy. (scale bars, 50 µm).

Discussion: The cationic control polypeptide **HDA-*b*-PHAG$_m$** internalizes very less in amount even though treating with 4 times more amount than di-block copolypeptide. There is no significant difference in internalization with and without mannan pre-treatment.

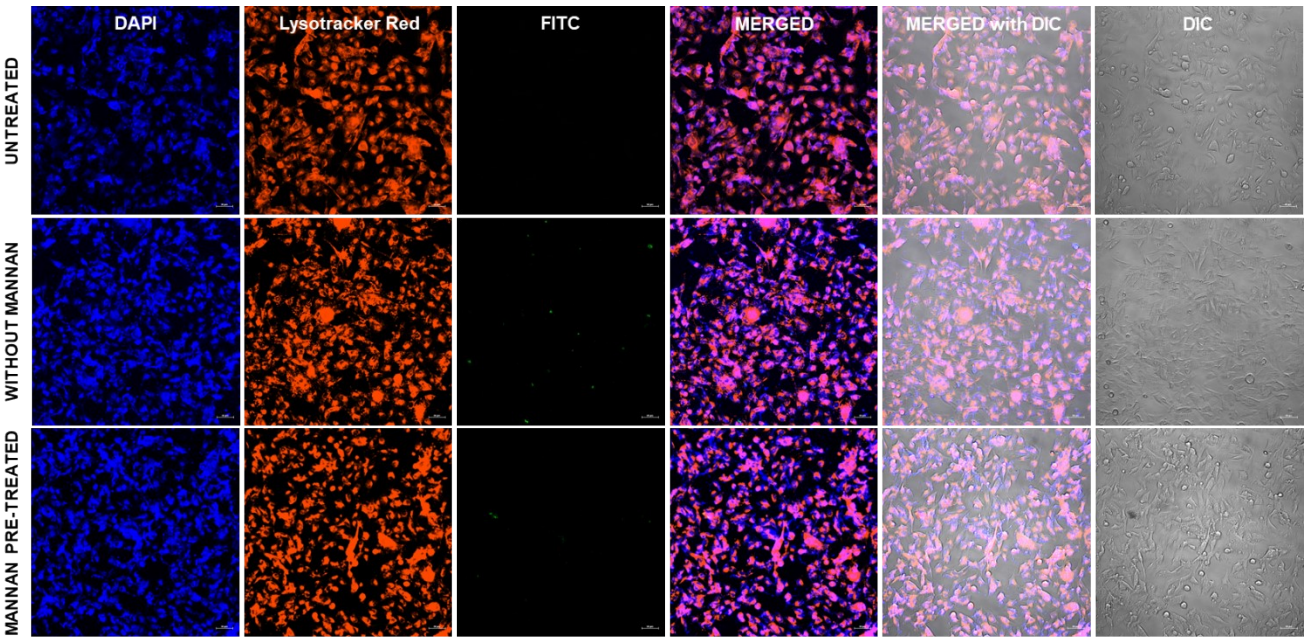


Figure S13: Cellular uptake experiment of fluorescein tagged **HDA-*b*-PHAG$_m$** on MDA-MB-231 cells for 4 h. MDA-MB-231 cells were cultured for 30 mins with mannan (300 µg) and then cultured for 4 h with **Fl_HDA-*b*-PHAG$_m$** (50 µg mL^{-1}) in DMEM and then stained with LysoTracker Red (50 nM) (for staining lysosomes) for 30 min and Hoechst 33342 (80 µM) (for staining nuclei) for 10 min. The cells were probed by confocal microscopy. (scale bars, 50 µm).

Discussion: The cationic control polypeptide **HDA-*b*-PHAG$_m$** internalizes very less in amount even though treating with 4 times more amount than di-block copolypeptide. There is no significant difference in internalization with and without mannan pre-treatment.

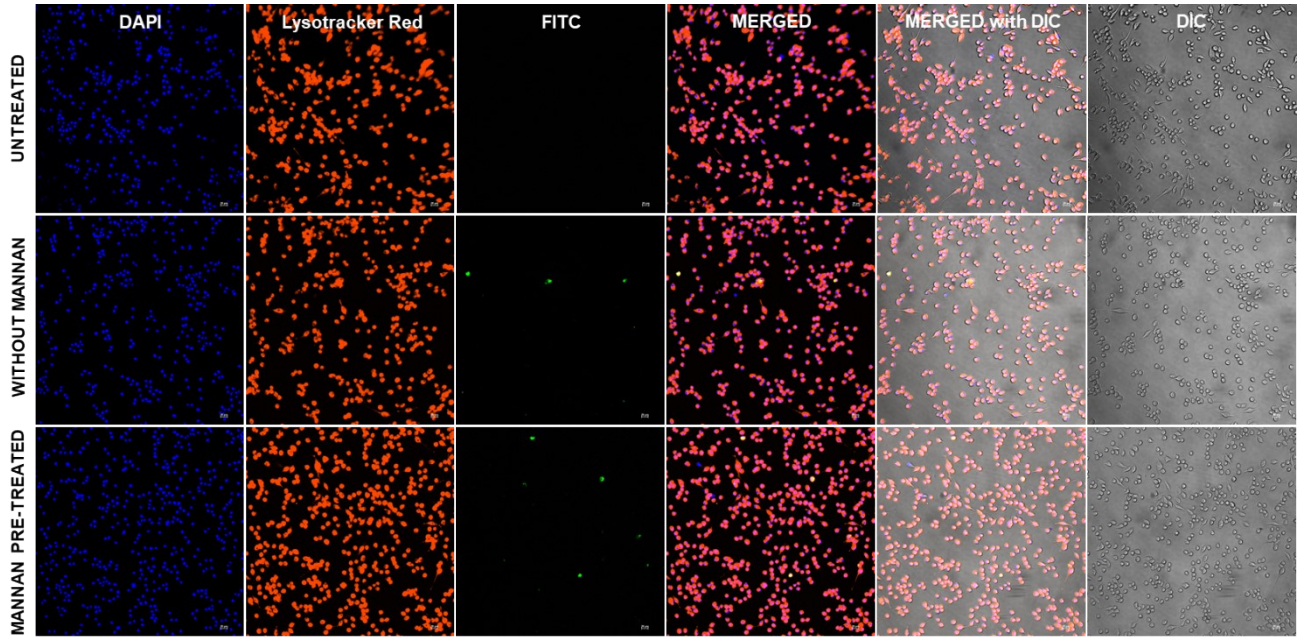


Figure S14: Cellular uptake experiment of fluorescein tagged **HDA-*b*-PHAG$_m$** on RAW 264.7 cells for 4 h. RAW 264.7 cells were cultured for 30 mins with mannan (300 µg) and then cultured for 4 h with **Fl_HDA-*b*-PHAG$_m$** (50 µg mL^{-1}) in DMEM and then stained with LysoTracker Red (50 nM) (for staining lysosomes) for 30 min and hoechst33342 (80 µM) (for staining nuclei) for 10 min. The cells were probed by confocal microscopy. (scale bars, 20 µm).

Discussion: The cationic control polypeptide **HDA-*b*-PHAG$_m$** internalizes very less in amount even though treating with 4 times more amount than di-block copolypeptide. There is no significant difference in internalization with and without mannan pre-treatment.

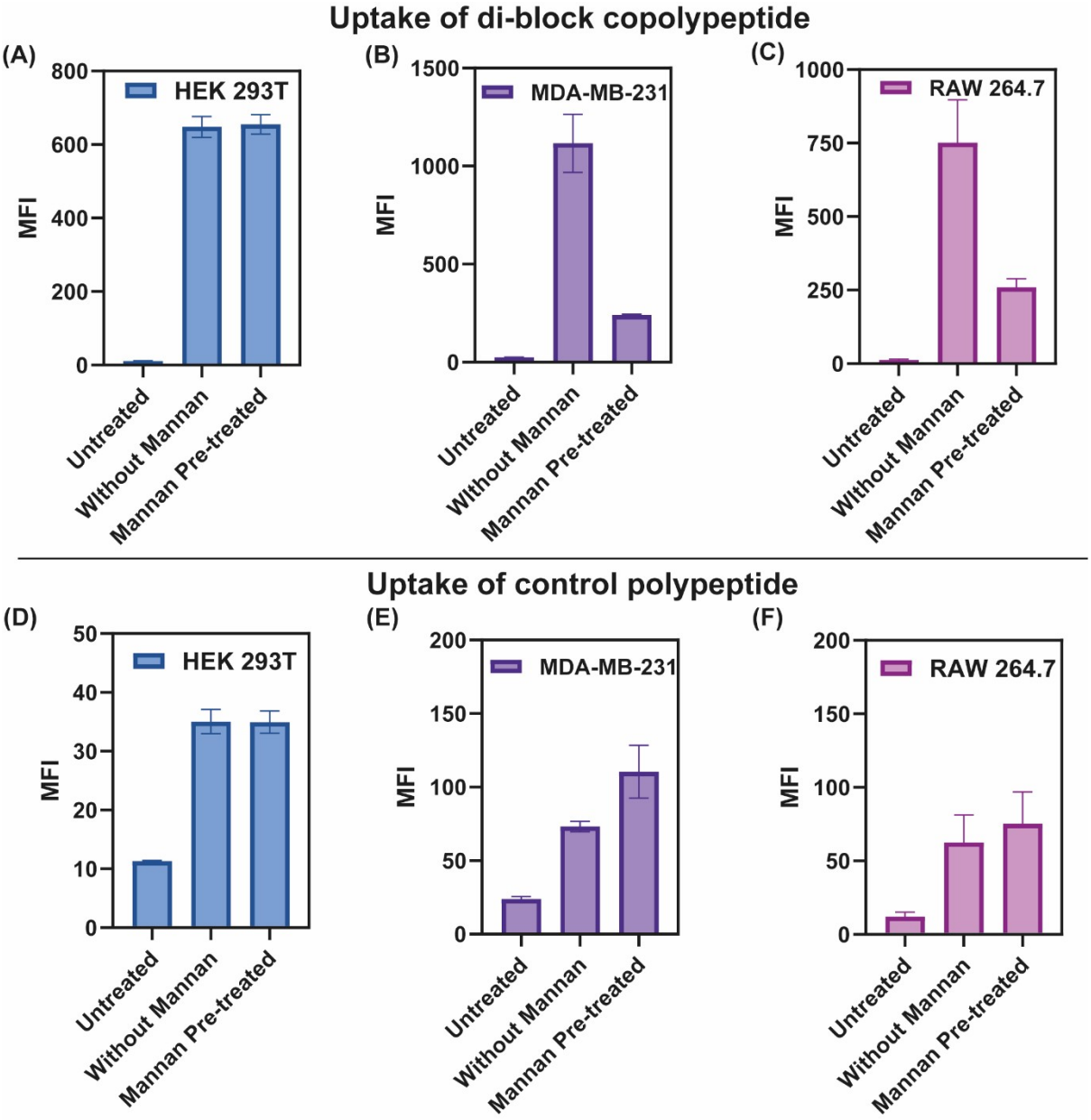


Figure S15: Flow cytometry assay for the cellular uptake of fluorescein tagged HDA-PHAG$_m$-PLSA$_n$ (block copolypeptide **Fl_HDA-PHAG$_m$-PLSA$_n$**) and control polypeptide **Fl_HDA-*b*-PHAG$_m$** on HEK 293T, MDA-MB-231, and RAW 264.7 cell lines for 4 h after mannan pre-treatment (300 µg for 30 mins). HEK 293T (A, D), MDA-MB-231 (B,E), and RAW 264.7 (C,F) cells were pretreated with 300 µg of

mannan for 30 minutes and then cultured for 4 h with block copolypeptide **Fl_HDA-PHAG$_m$-PLSA$_n$** (100 µg mL^{-1}) & control polypeptide **Fl_HDA-*b*-PHAG$_m$** (100 µg mL^{-1}) in DMEM.

Discussion: The internalization of cationic di-block copolypeptide reduced after mannan treatment for MDA-MB-231 and RAW 264.7 cells, but stayed intact for HEK 293T cells. The internalization of control polypeptide **HDA-*b*-PHAG$_m$** was unchanged even after mannan pre-treatment. This proves that the di-block copolypeptides enter the cells via mannose receptor-mediated pathway while the control polypeptides have no role in receptor-mediated endocytosis.

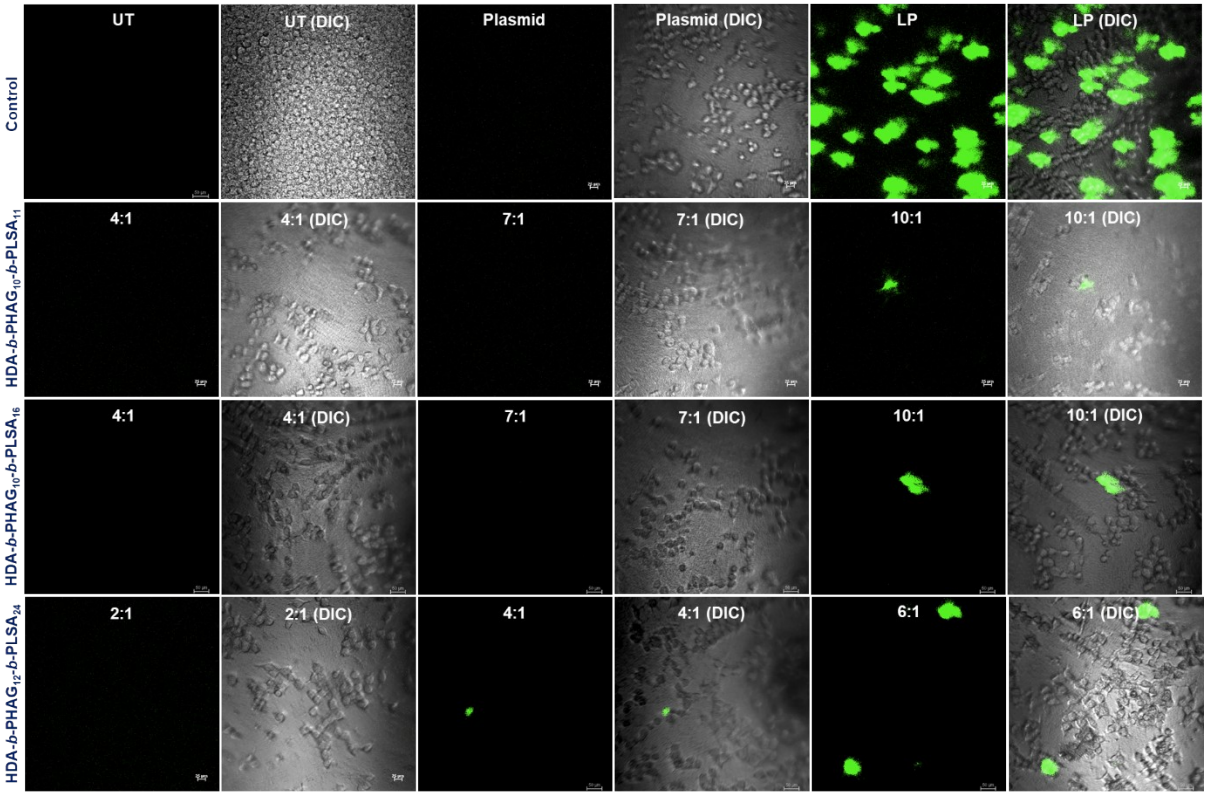


Fig S16: Transfection experiment (eGFP expression) in HEK 293T cells for the treatment of polyplexes at different charge ratio.

Discussion: Commercial reagent Lipofectamine 2000 showed good transfection of eGFP, but all other polyplexes at different charge ratio showed very little transfection of eGFP.

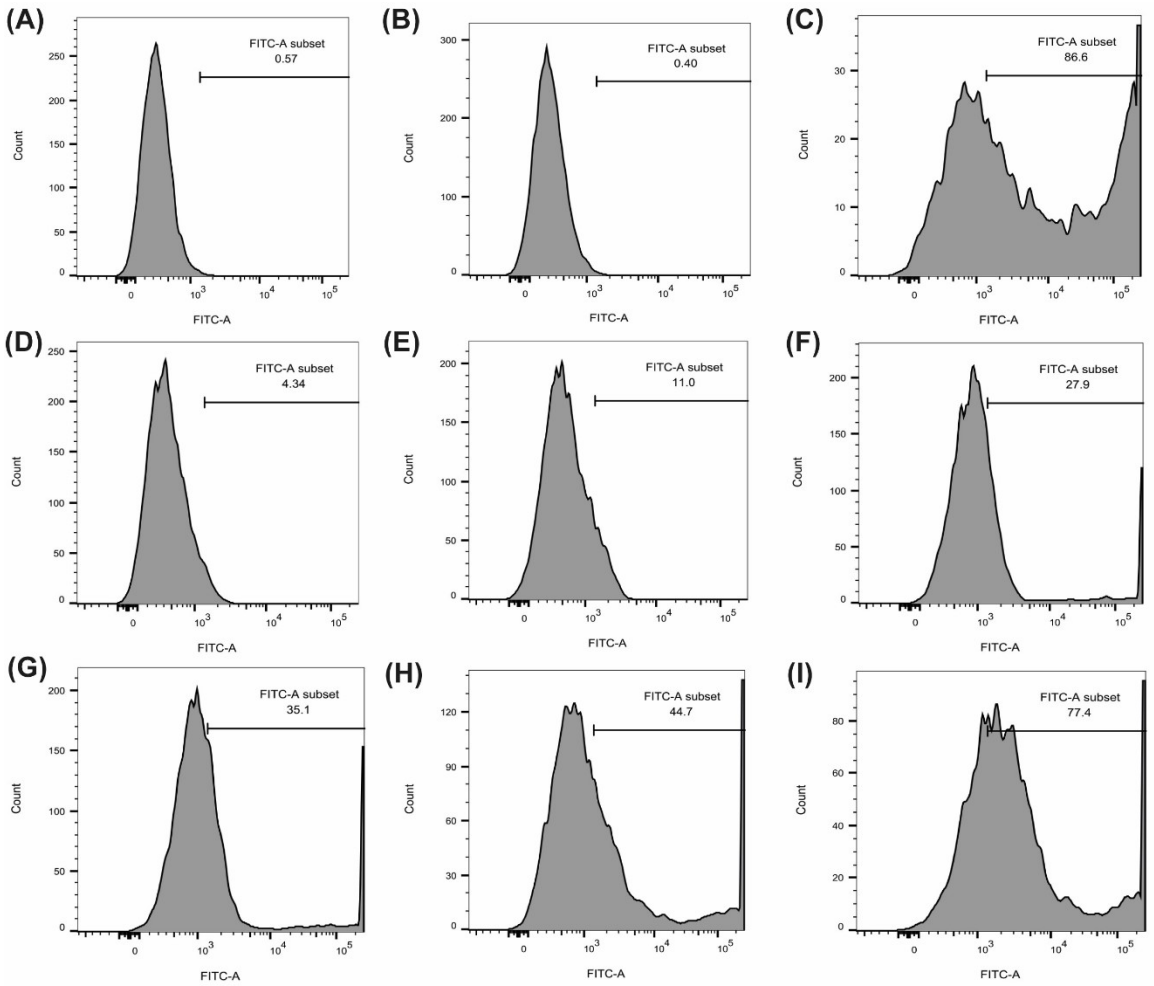


Fig S17: Transfection experiment (eGFP expression) in MDA-MB-231 cells for the treatment of polyplexes at different charge ratio. (A) untreated, (B) naked plasmid, (C) Lipofectamine 2000, (D) HDA-*b*-$PHAG_{10}$-*b*-$PLSA_{11}$ (CR 7), (E) HDA-*b*-$PHAG_{10}$-*b*-$PLSA_{11}$ (CR 10), (F) HDA-*b*-$PHAG_{10}$-*b*-$PLSA_{16}$ (CR 7), (G) HDA-*b*-$PHAG_{10}$-*b*-$PLSA_{16}$ (CR 10), (H) HDA-*b*-$PHAG_{12}$-*b*-$PLSA_{24}$ (CR 4), (I) HDA-*b*-$PHAG_{12}$-*b*-$PLSA_{24}$ (CR 6).

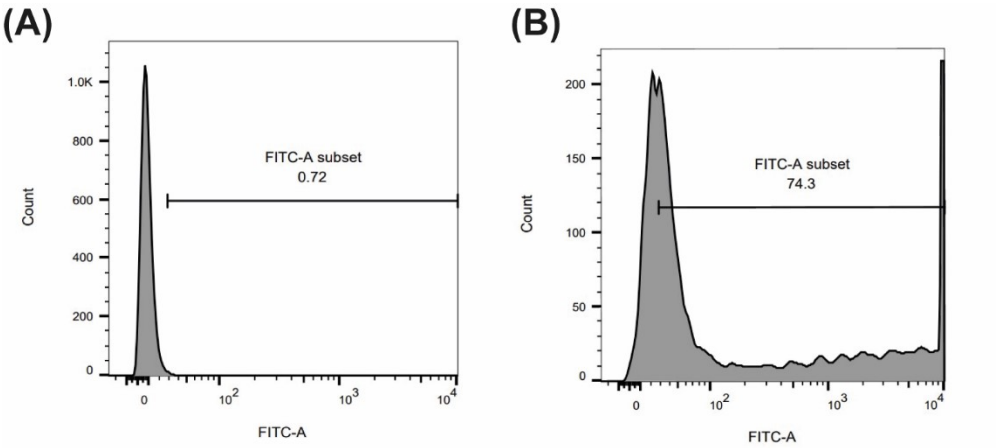


Fig S18: Transfection experiment (eGFP expression) in HEK 293T cells for the treatment of (A) untreated, (B) Lipofectamine 2000. (Note: the polypeptides at different charge ratios showed very little transfection).

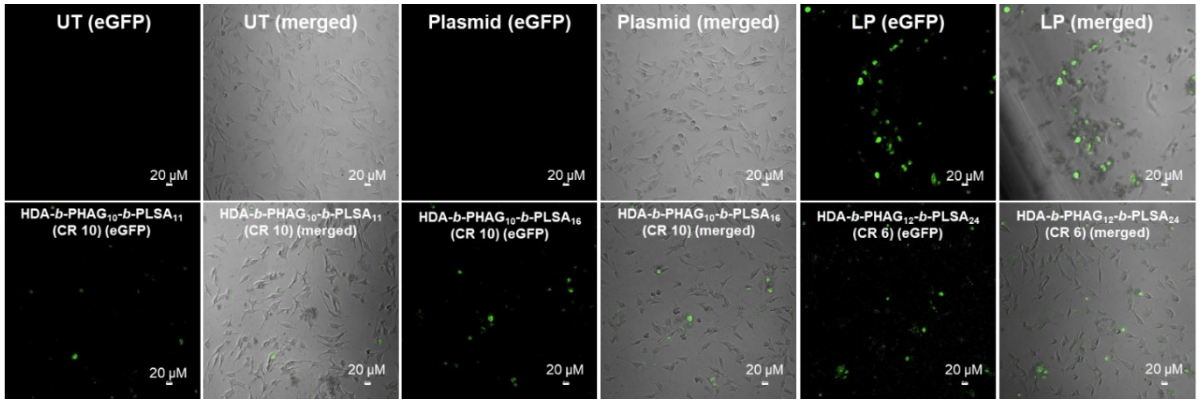


Figure S19: Transfection experiment (eGFP expression) in MDA-MB-231 cells for the treatment of polyplexes at different charge ratios in presence of serum containing media (DMEM). Control groups are taken as Untreated, only plasmid, and Lipofectamine 2000.

Discussion: Polyplexes were formulated as written in the experimental section and then diluted with serum containing media and treated to the cells for 24 hours. After 24 h, incubated media was replaced with fresh serum containing media. This experiment was conducted to investigate the performance in transfection of the polypeptides in presence of total serum containing media. We observed the same trend for the three polypeptides, that is HDA-*b*-$PHAG_{12}$-*b*-$PLSA_{24}$ is the most effective and HDA-*b*-$PHAG_{10}$-*b*-$PLSA_{11}$ is the least effective. It is evident that the transfection efficiency gets reduced a bit in presence of total serum containing media (even in the case of Lipofectamine 2000).

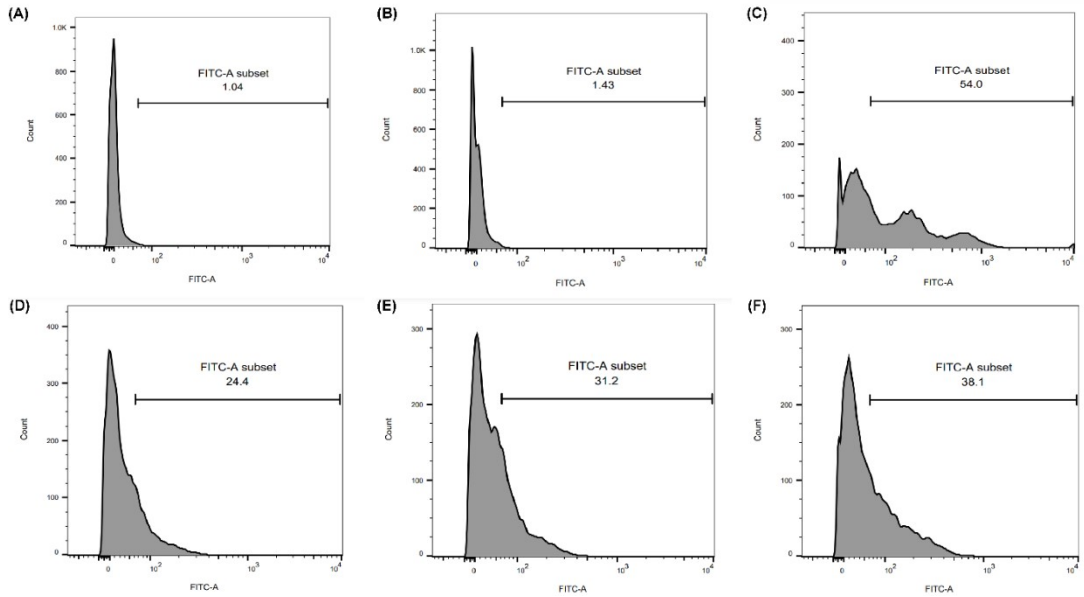


Fig S20: Transfection experiment (eGFP expression) in MDA-MB-231 cells for the treatment of polyplexes in presence of serum containing media (DMEM); (A) untreated, (B) only plasmid, (C) Lipofectamine 2000, (D) HDA-*b*-$PHAG_{10}$-*b*-$PLSA_{11}$ (CR 10), (E) HDA-*b*-$PHAG_{10}$-*b*-$PLSA_{16}$ (CR 10), (F) HDA-*b*-$PHAG_{12}$-*b*-$PLSA_{24}$ (CR 6).

Discussion: Polyplexes were formulated as written in the experimental section and then diluted with serum containing media and treated to the cells for 24 hours. After 24 h, incubated media was replaced with fresh serum containing media. This experiment was conducted to investigate the performance in transfection of the polypeptides in presence of total serum containing media. AFtedr 48 h, we observed the same trend for the three polypeptides, that is HDA-*b*-$PHAG_{12}$-*b*-$PLSA_{24}$ is the most effective and HDA-*b*-$PHAG_{10}$-*b*-$PLSA_{11}$ is the least effective from flow cytometry analysis.

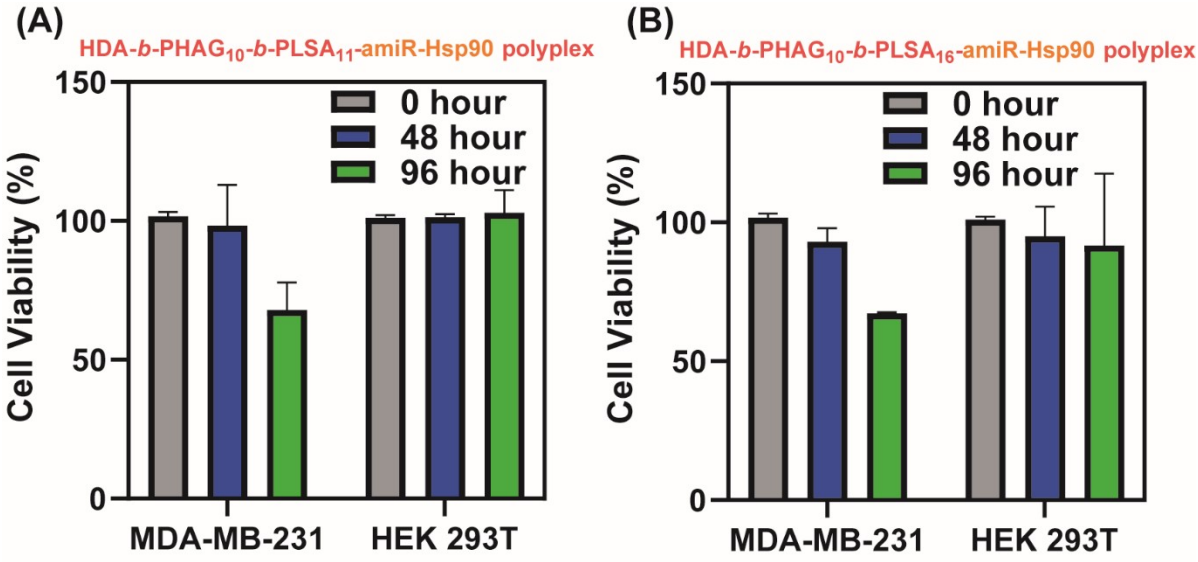


Fig S21: Cytotoxicity effect after transfection of amiR-Hsp90 plasmid in MDA-MB-231, and HEK 293T cells using (A) HDA-*b*-$PHAG_{10}$-*b*-$PLSA_{11}$ and (B) HDA-*b*-$PHAG_{10}$-*b*-$PLSA_{16}$ di-block copolypeptide showing cancer cell specific cell killing.

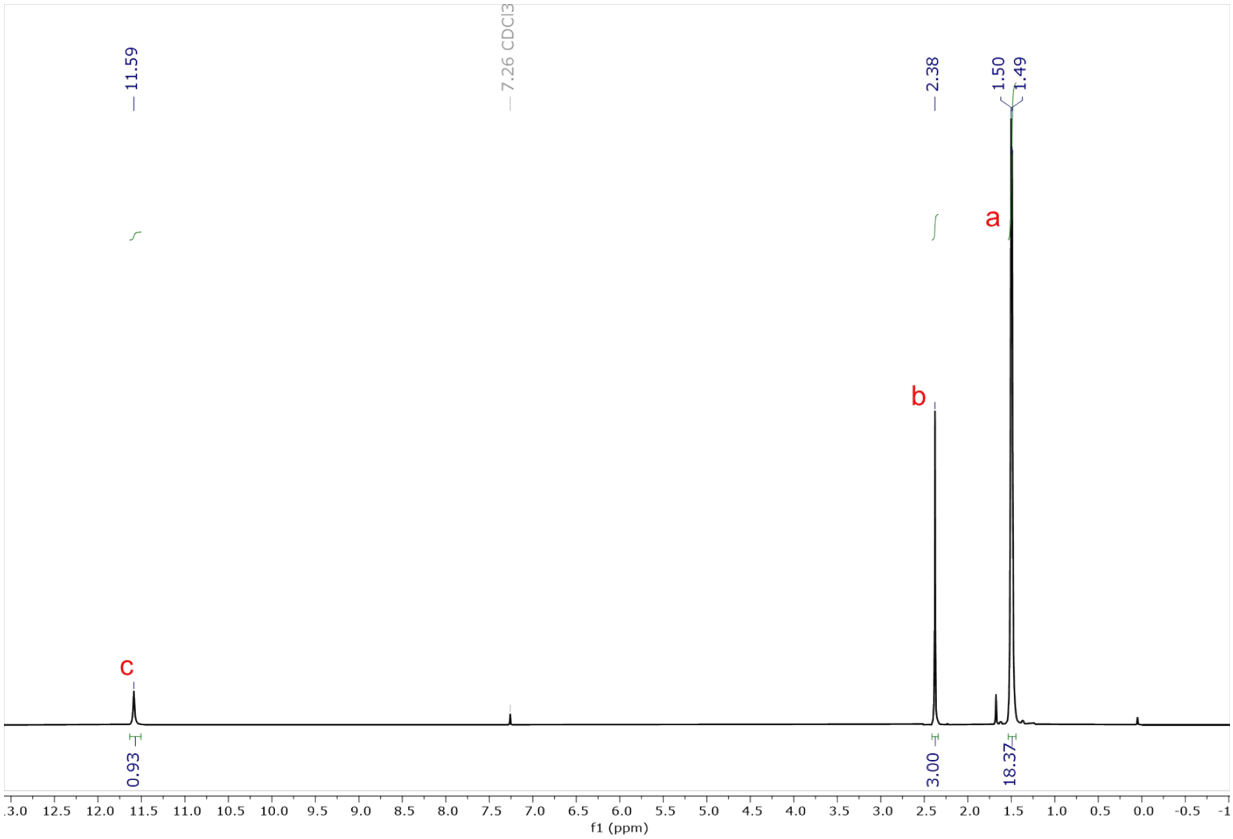


Figure S22: ^{1}H NMR of N,N'-di(Boc) guanidium hemisulfate

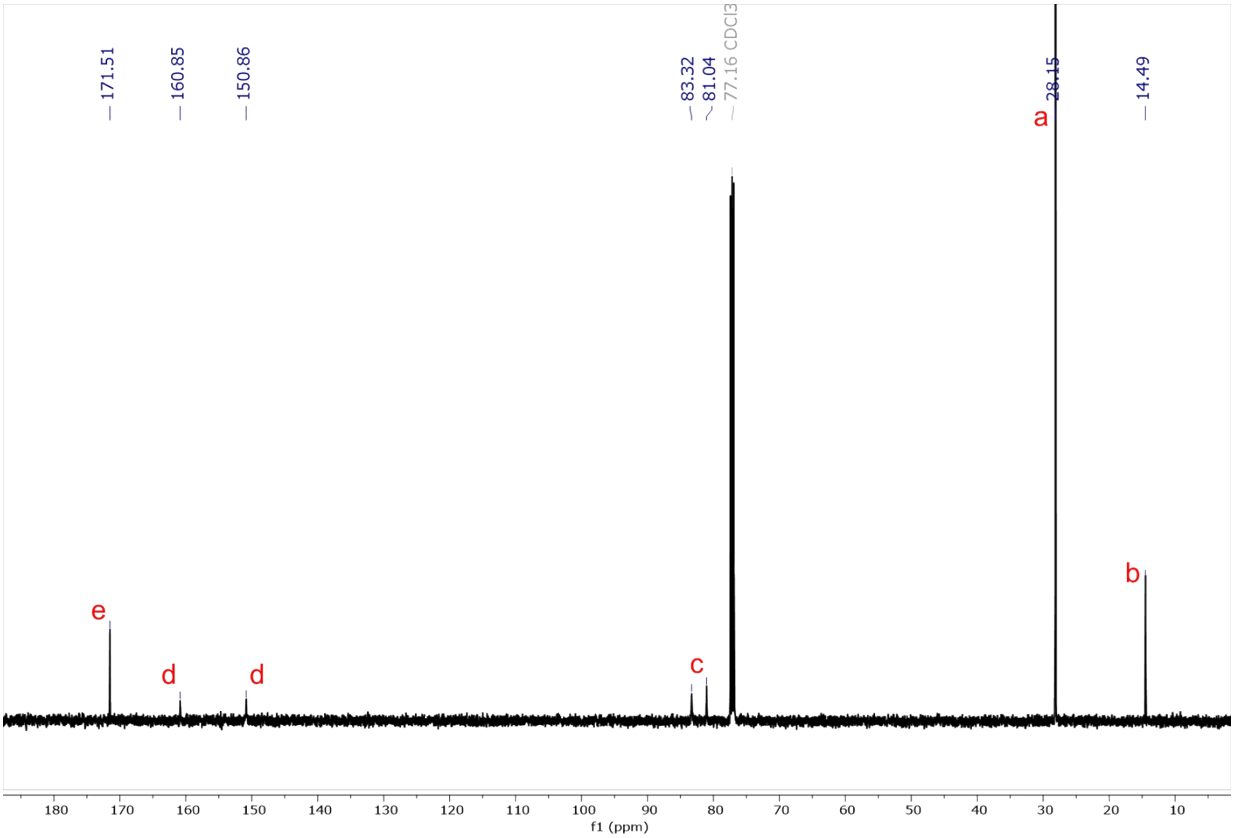


Figure S23: ^{13}C NMR of N,N'-di(Boc) guanidium hemisulfate.

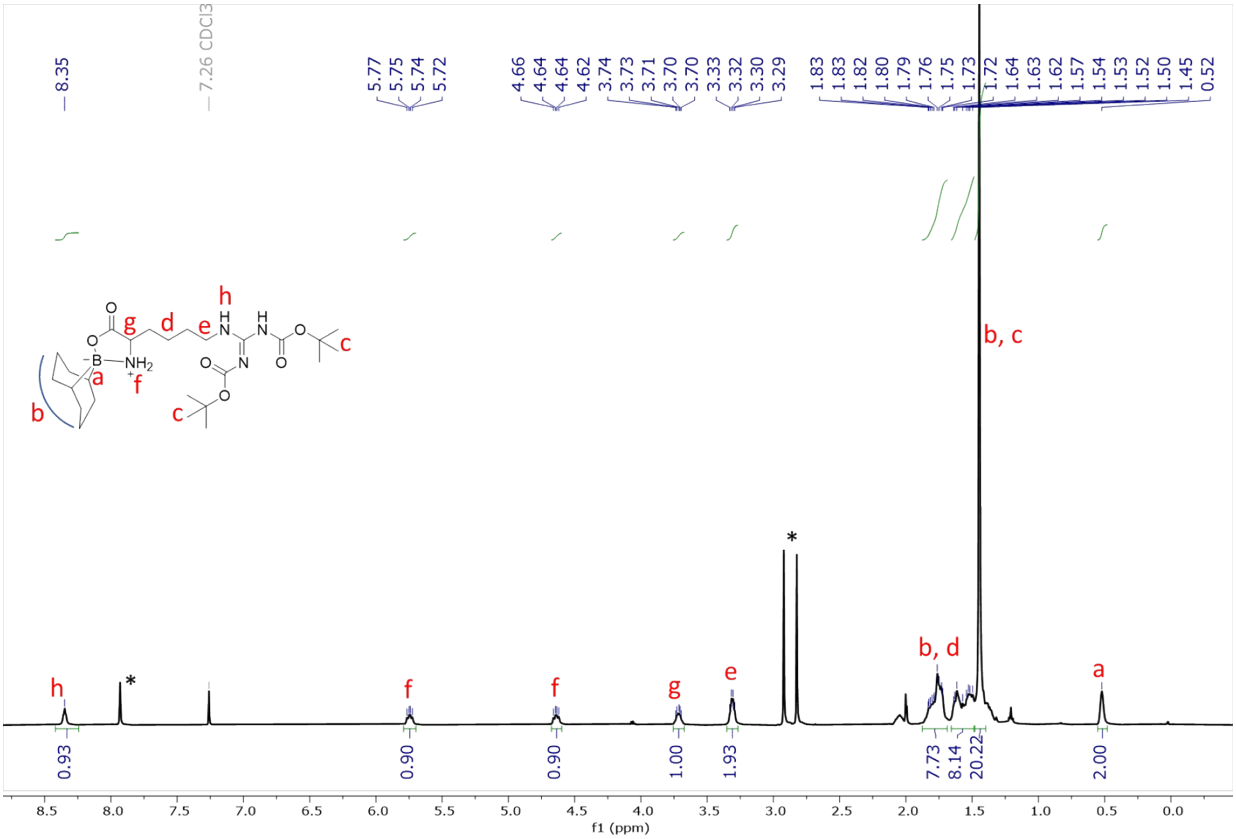


Figure S24: ^{1}H NMR of N,N´-di(Boc)-L-homoarginine 9-BBN complex

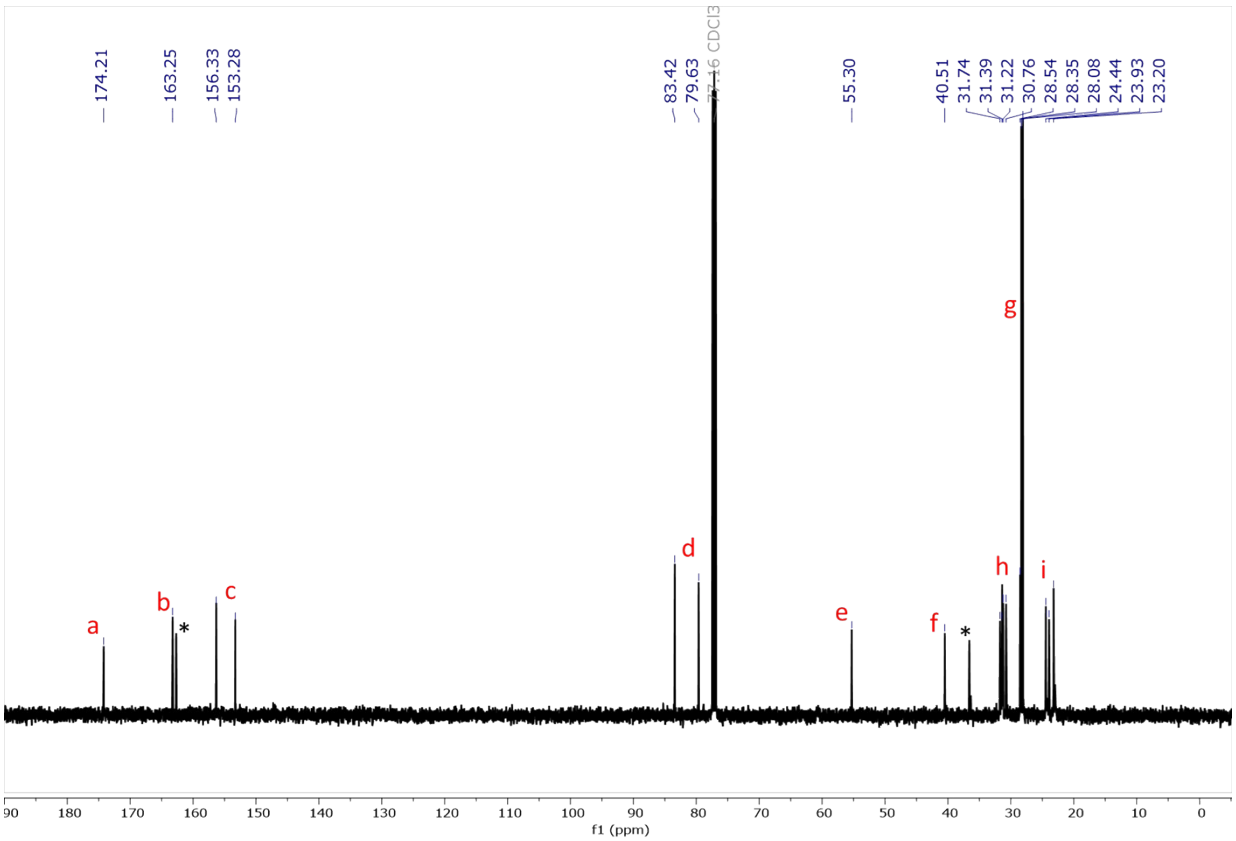


Figure S25: ^{13}C NMR of N,N´-di(Boc)-L-homoarginine 9-BBN complex

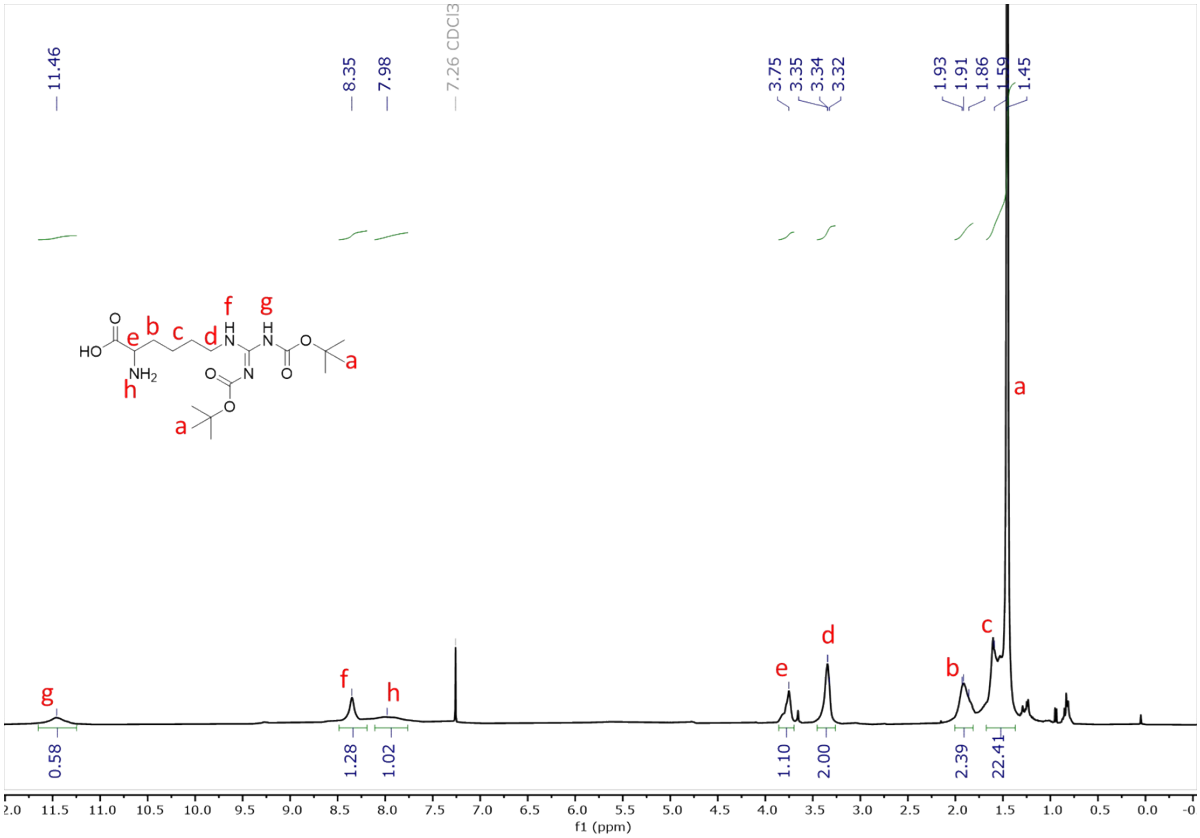


Figure S26: ^{1}H NMR of N,N´-di(Boc)-L-homoarginine

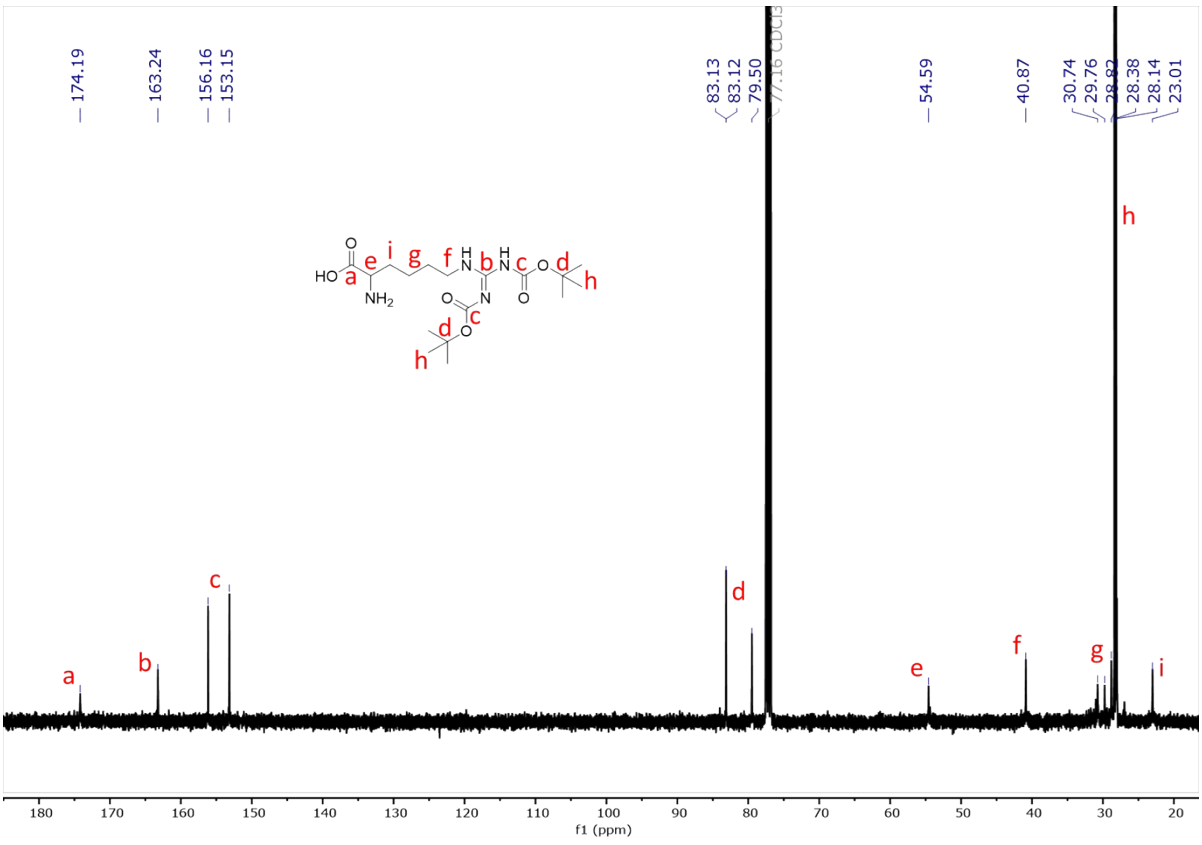


Figure S27: ^{13}C NMR of N,N´-di(Boc)-L-homoarginine

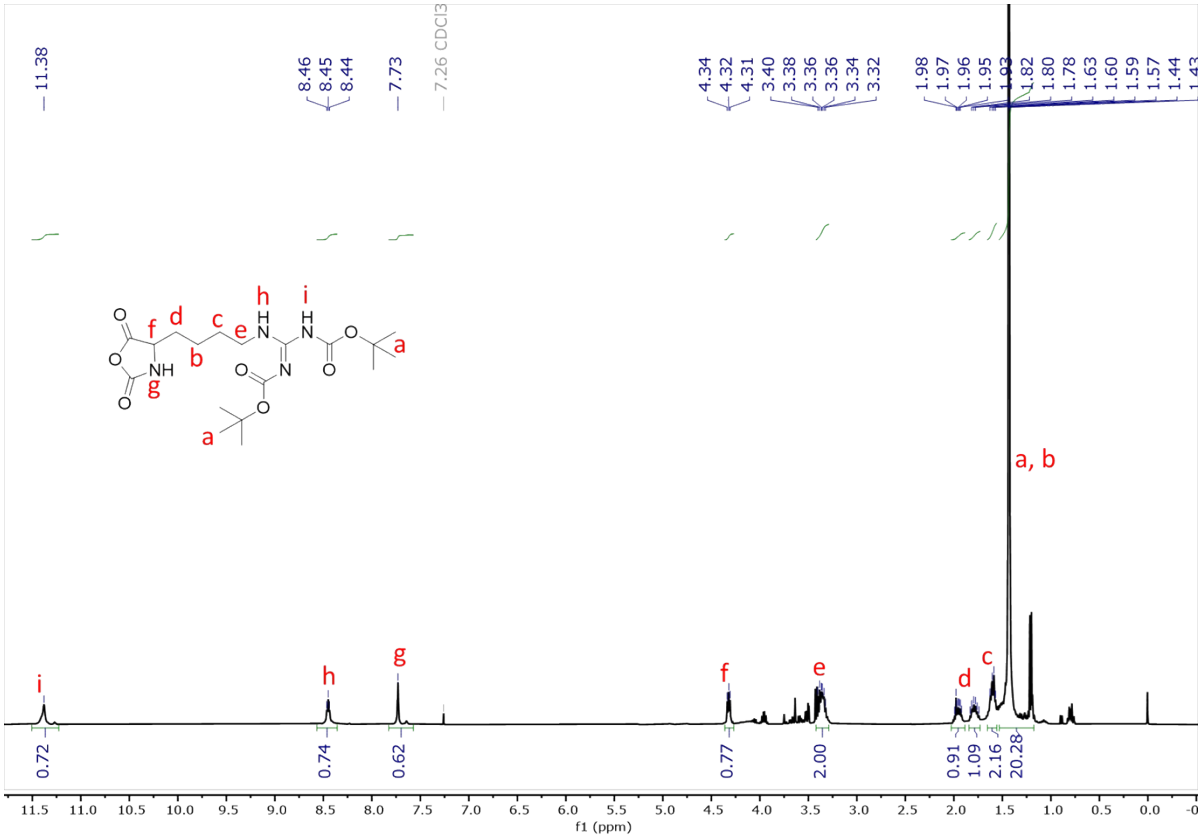


Figure S28: ^{1}H NMR of N,N´-di(Boc)-L-homoarginine-NCA (HAG-NCA)

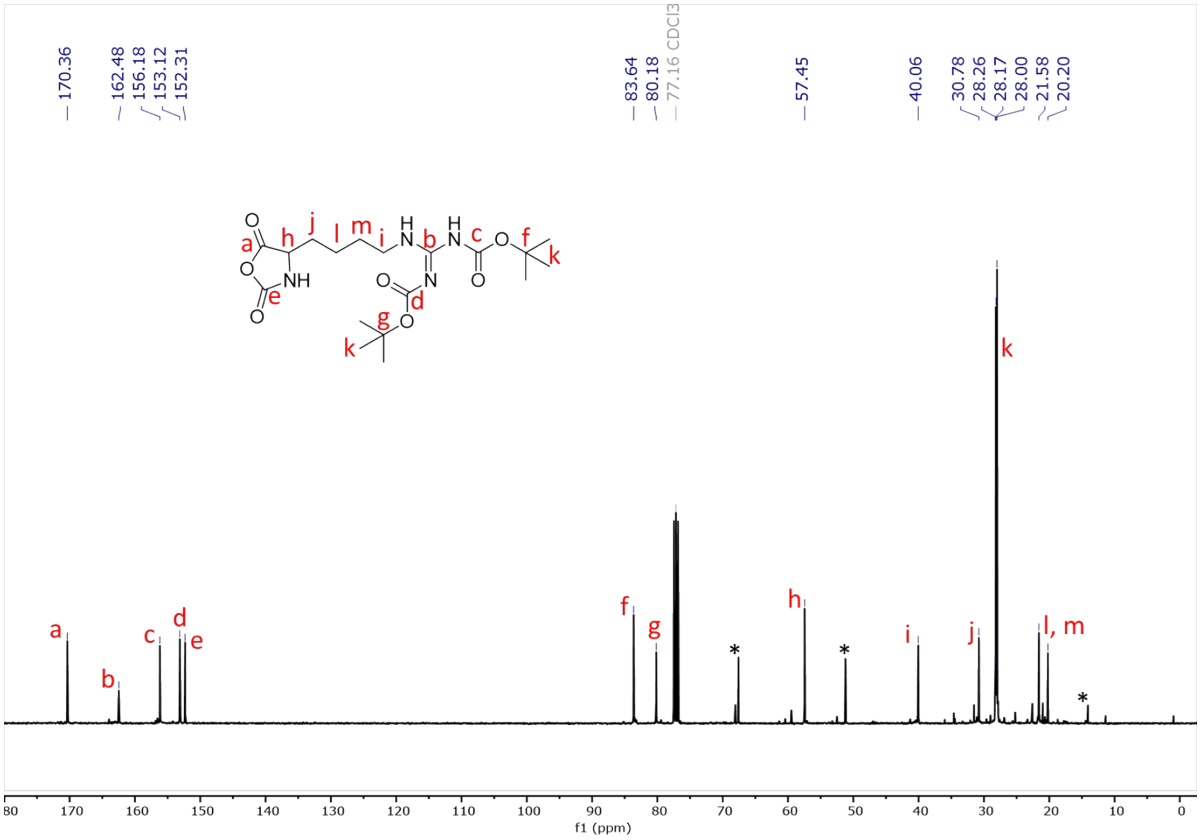


Figure S29: ^{13}C NMR of N,N´-di(Boc)-L-homoarginine-NCA (HAG-NCA)

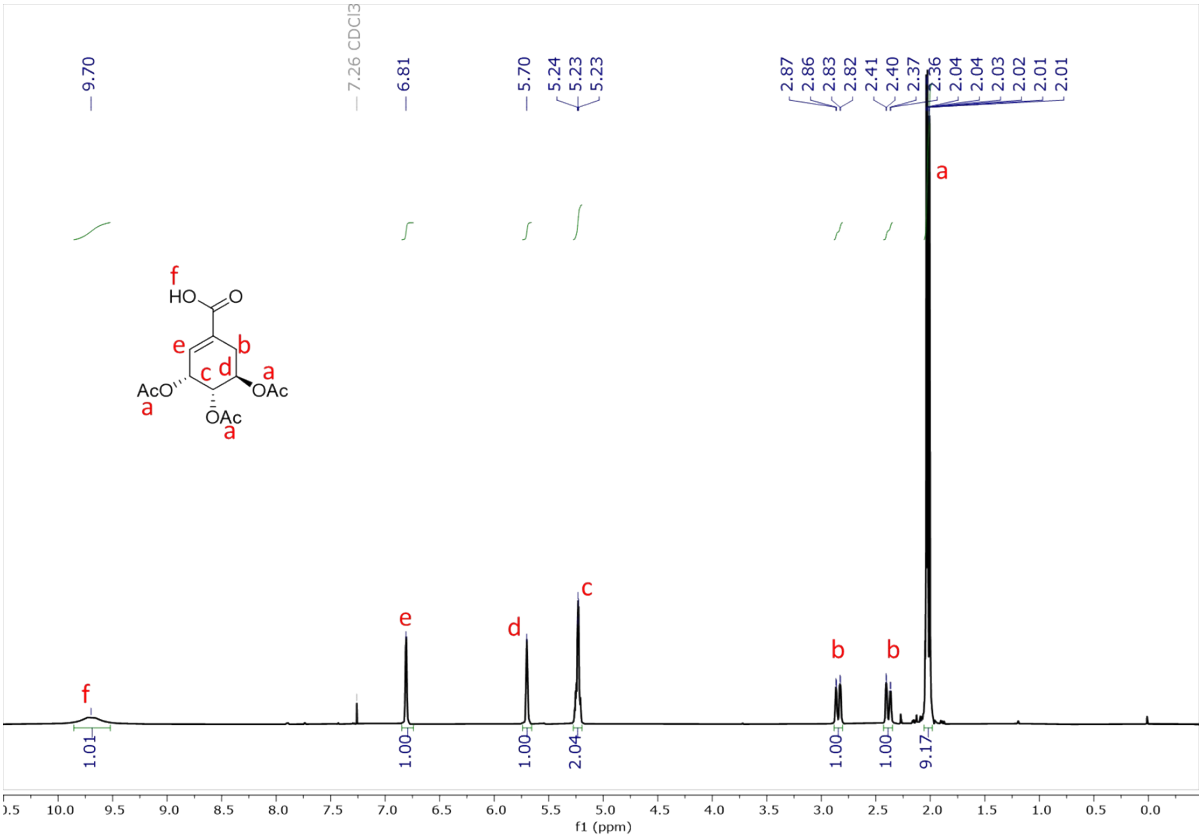


Figure S30: ^{1}H NMR of tri-acetyl shikimic acid

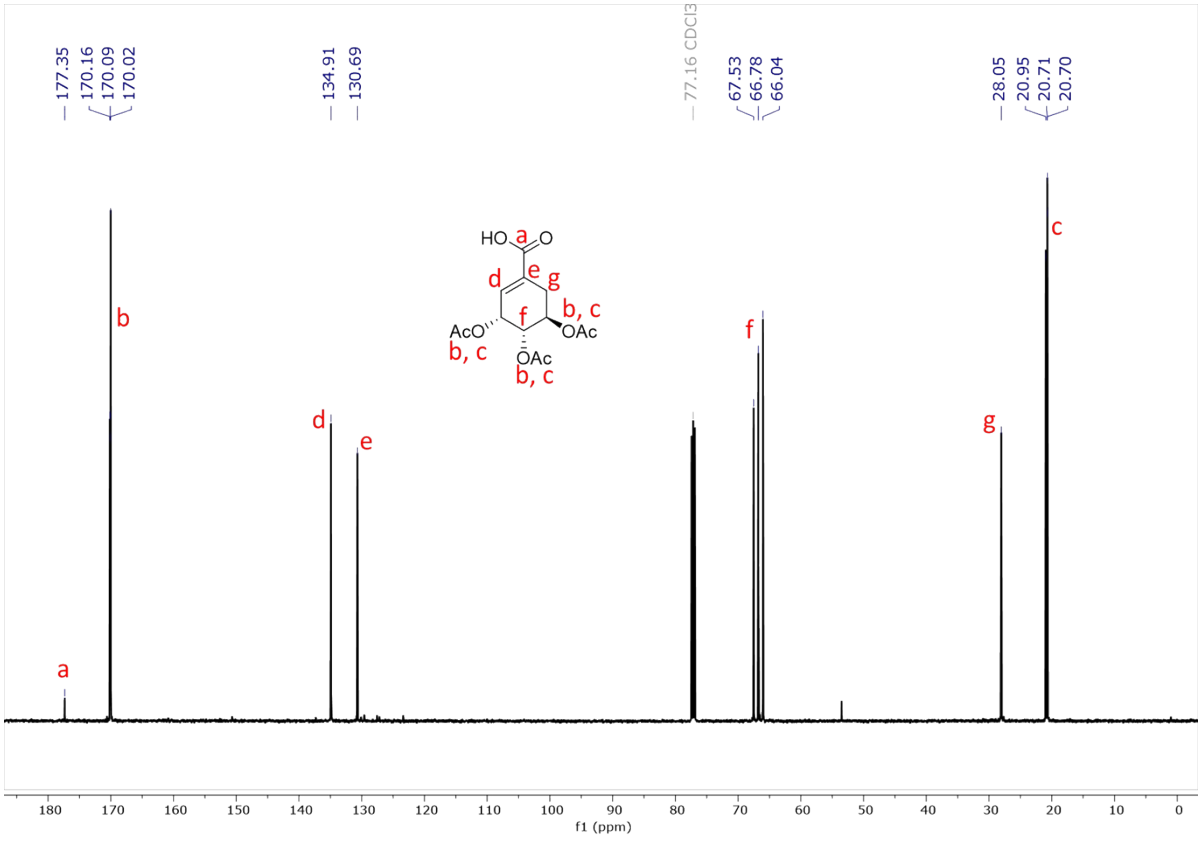


Figure S31: ^{13}C NMR of tri-acetyl shikimic acid

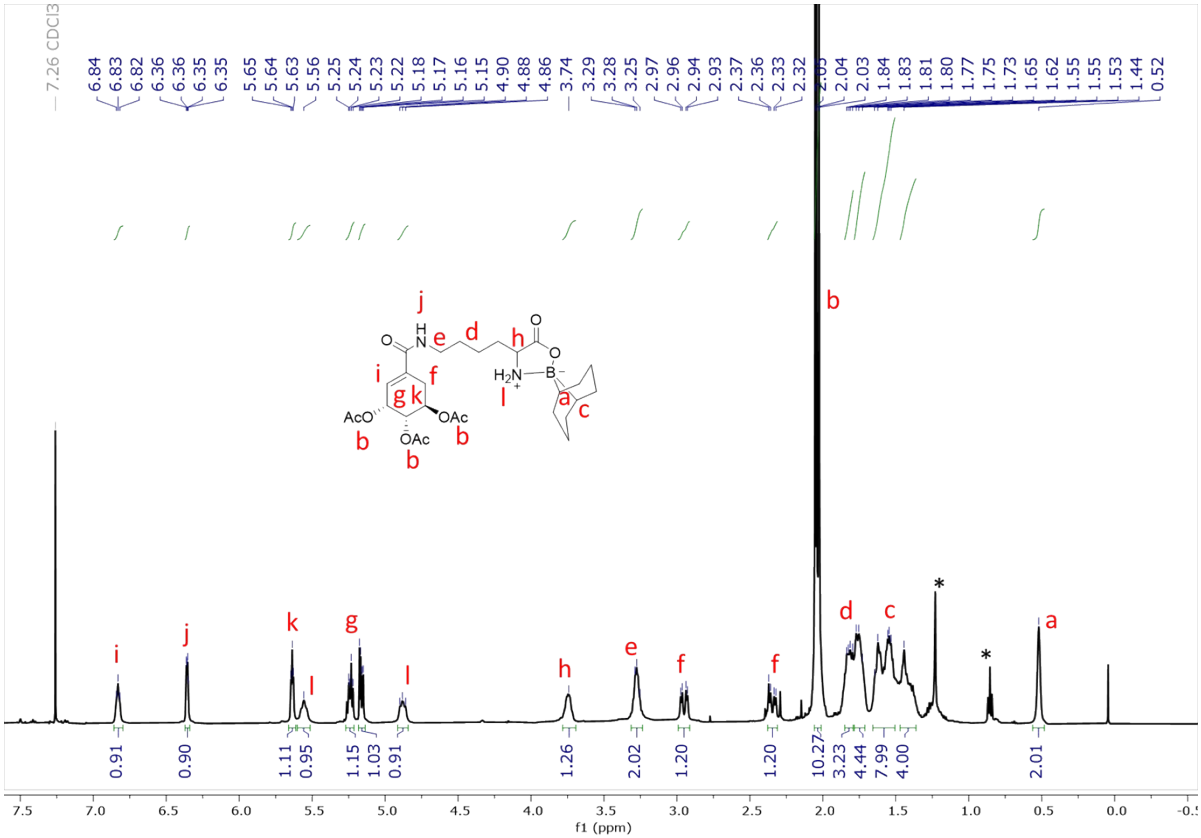


Figure S32: ^{1}H NMR of 9-BBN lysine-tri-acetyl shikimic complex

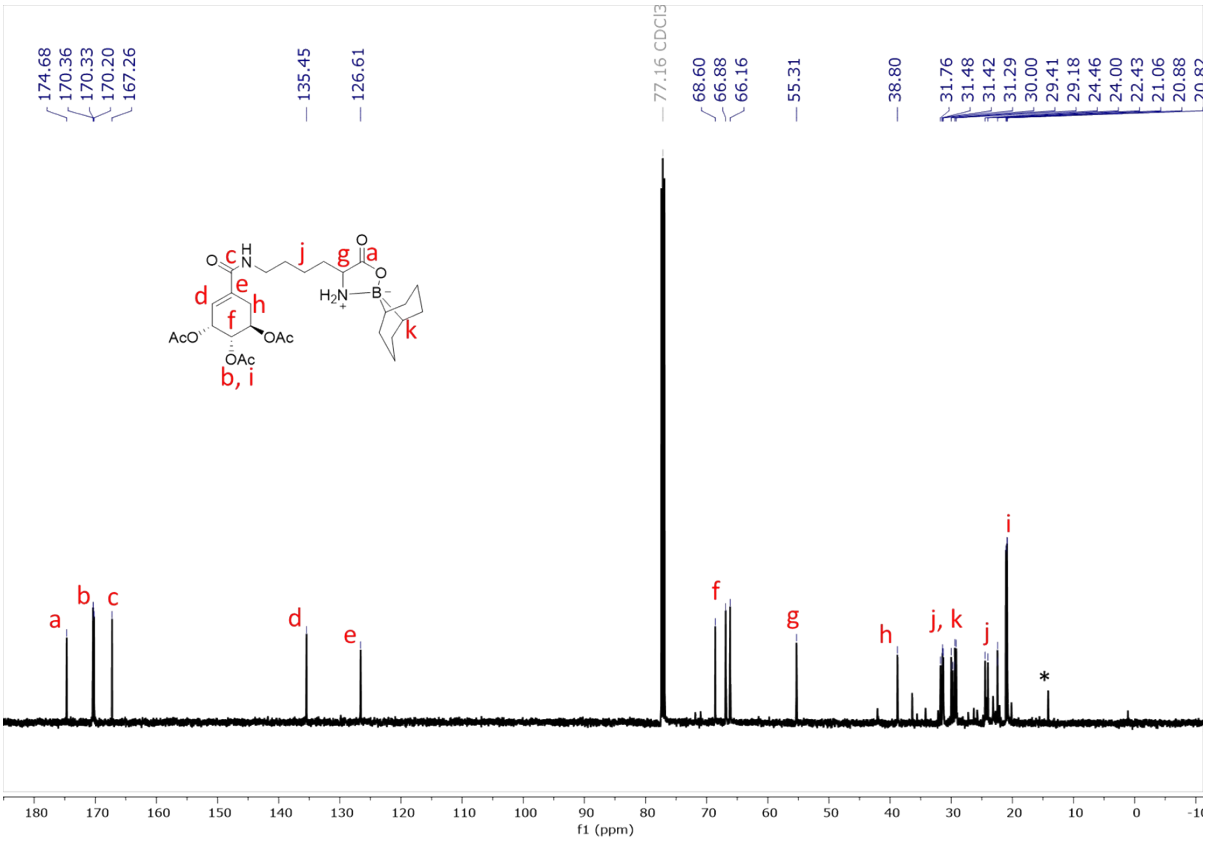


Figure S33: ^{13}C NMR of 9-BBN lysine-tri-acetyl shikimic complex

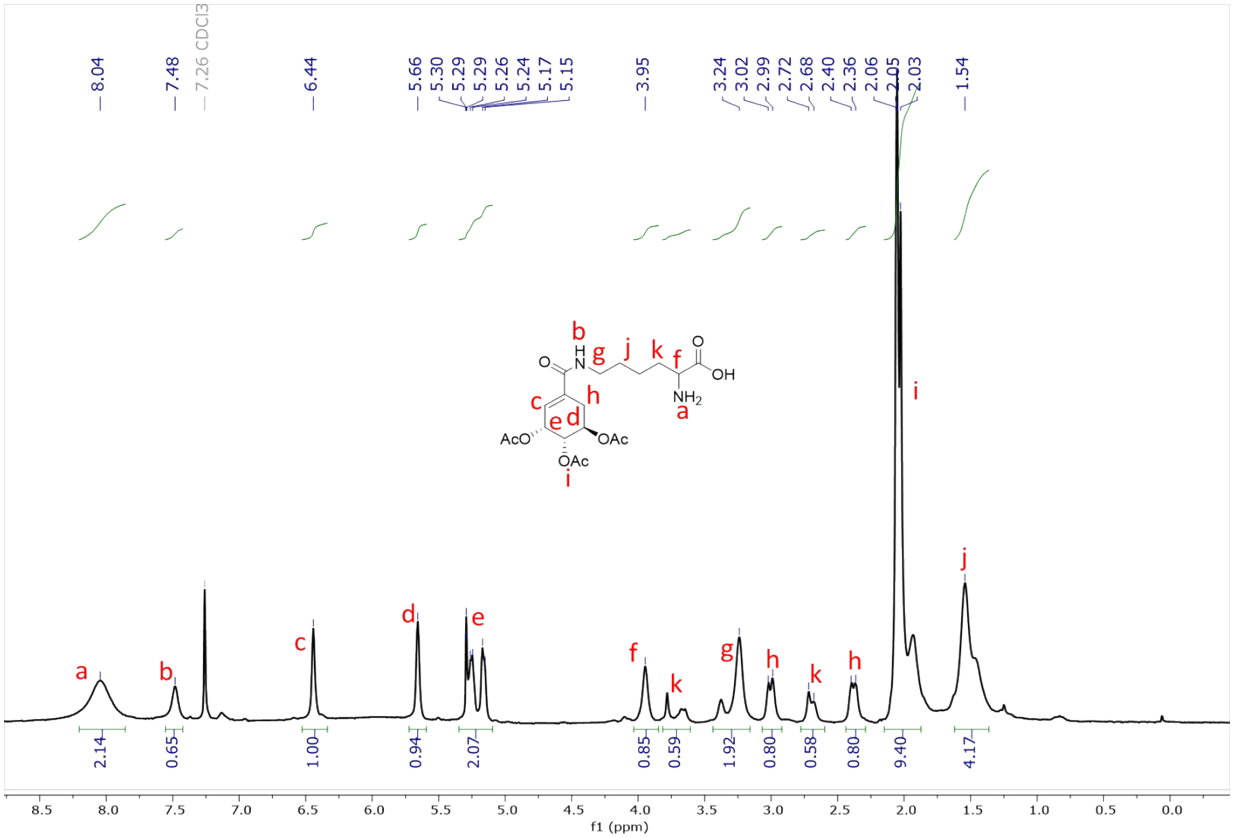


Figure S34: ^{1}H NMR of 9-BBN deprotected lysine-tri-acetyl shikimic (LSA) complex

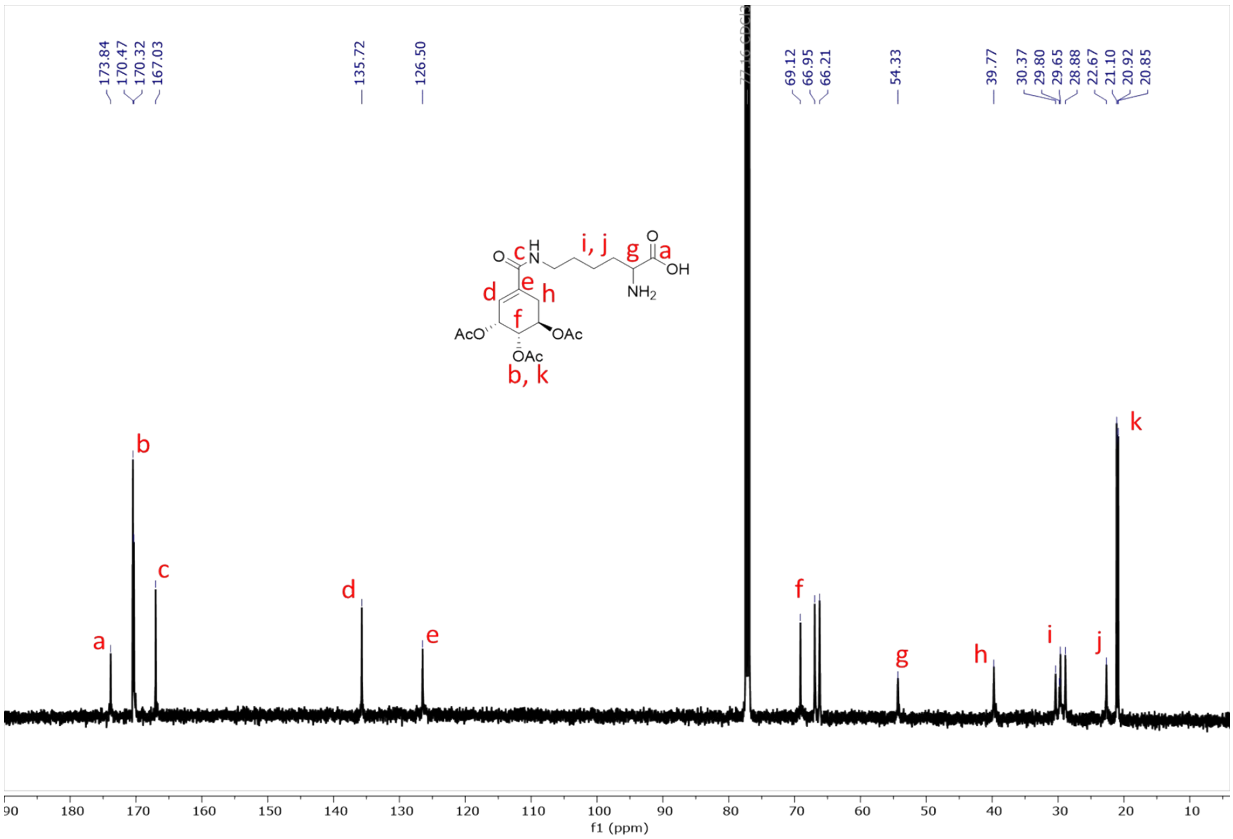


Figure S35: ^{13}C NMR of 9-BBN deprotected lysine-tri-acetyl shikimic (LSA) complex

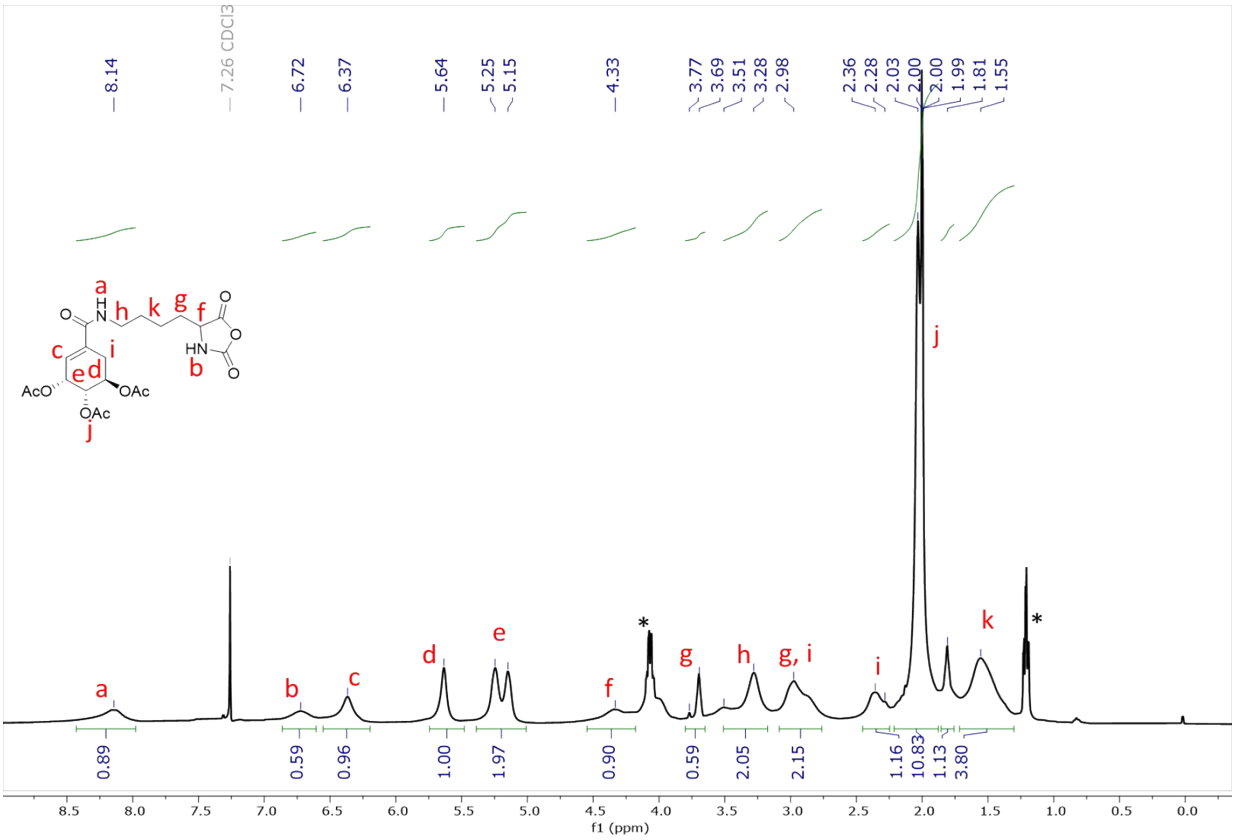


Figure S36: ^{1}H NMR of lysine-tri-acetyl shikimic (LSA) NCA

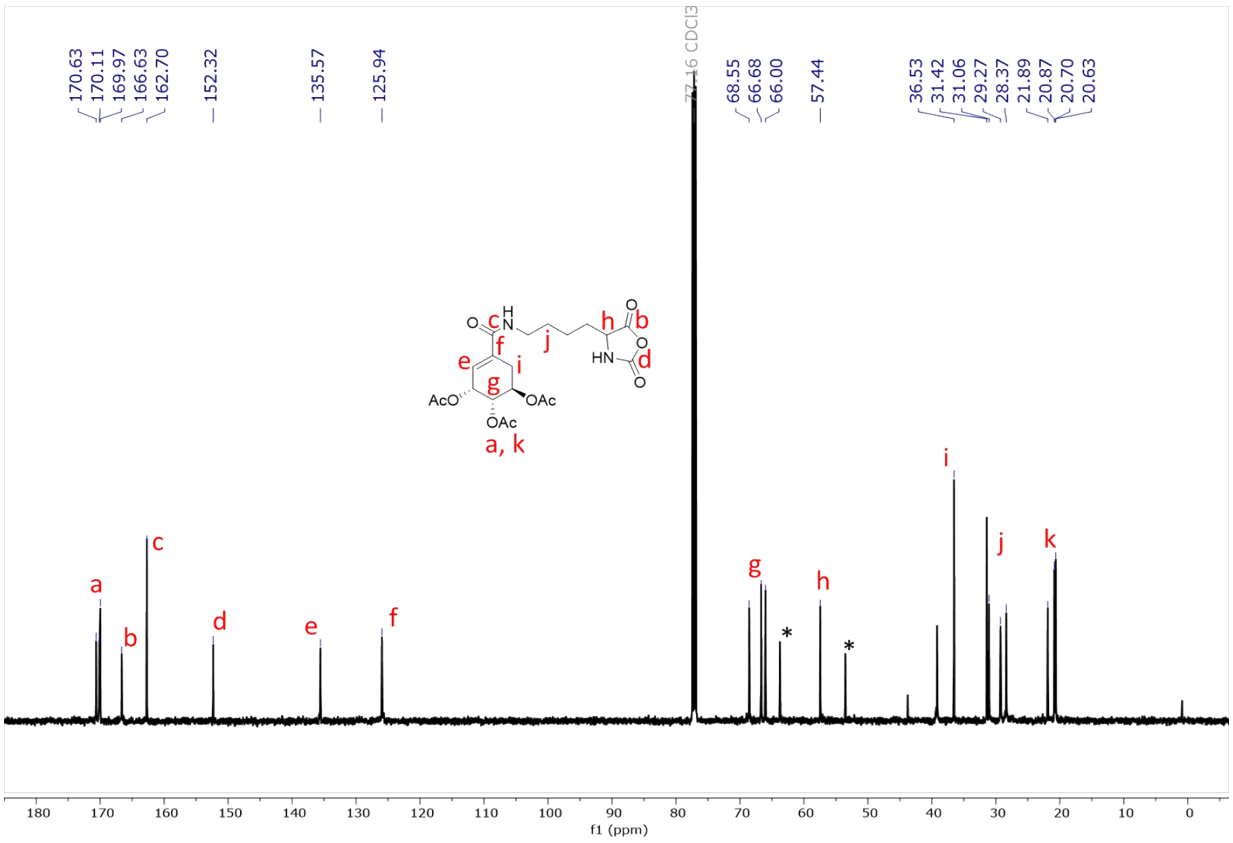


Figure S37: ^{13}C NMR of lysine-tri-acetyl shikimic (LSA) NCA

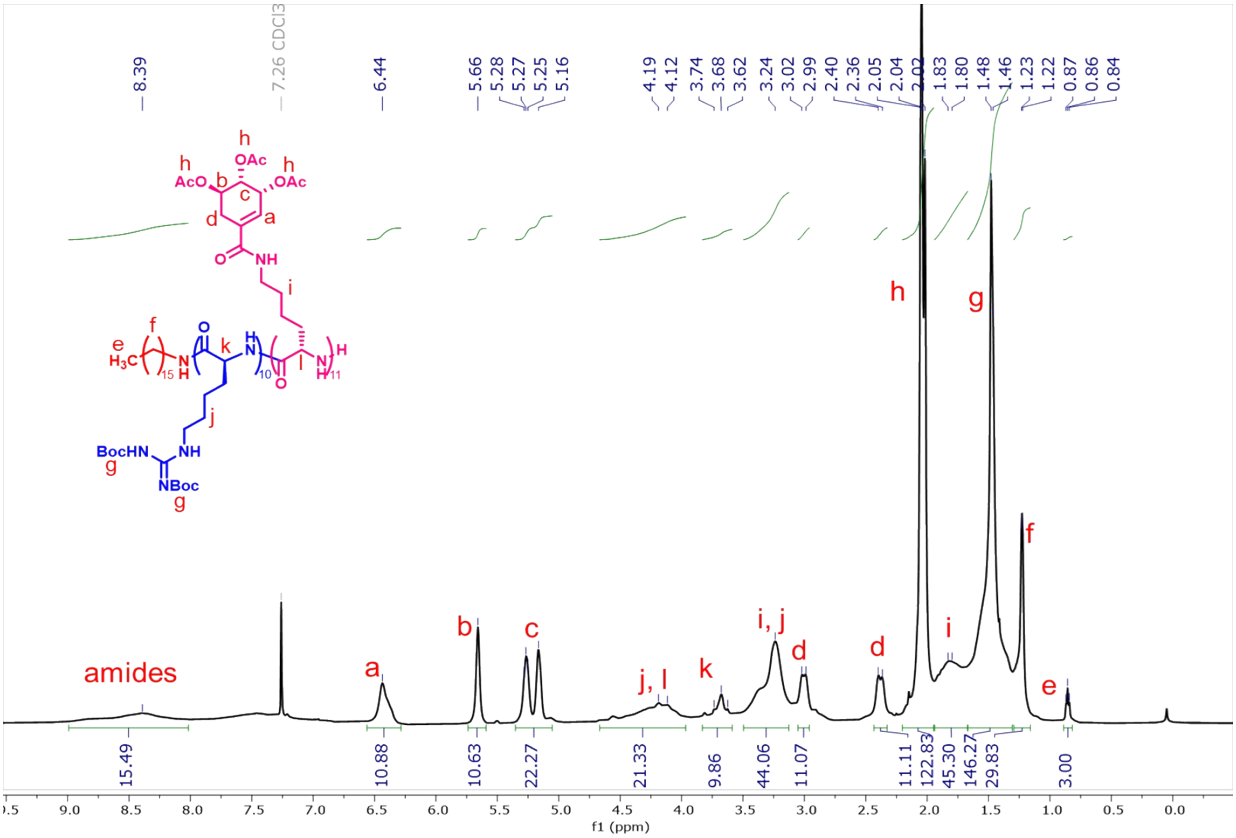


Figure S38: ^{1}H NMR of fully protected HDA-*b*-PHAG$_{10}$-*b*-PLSA$_{11}$

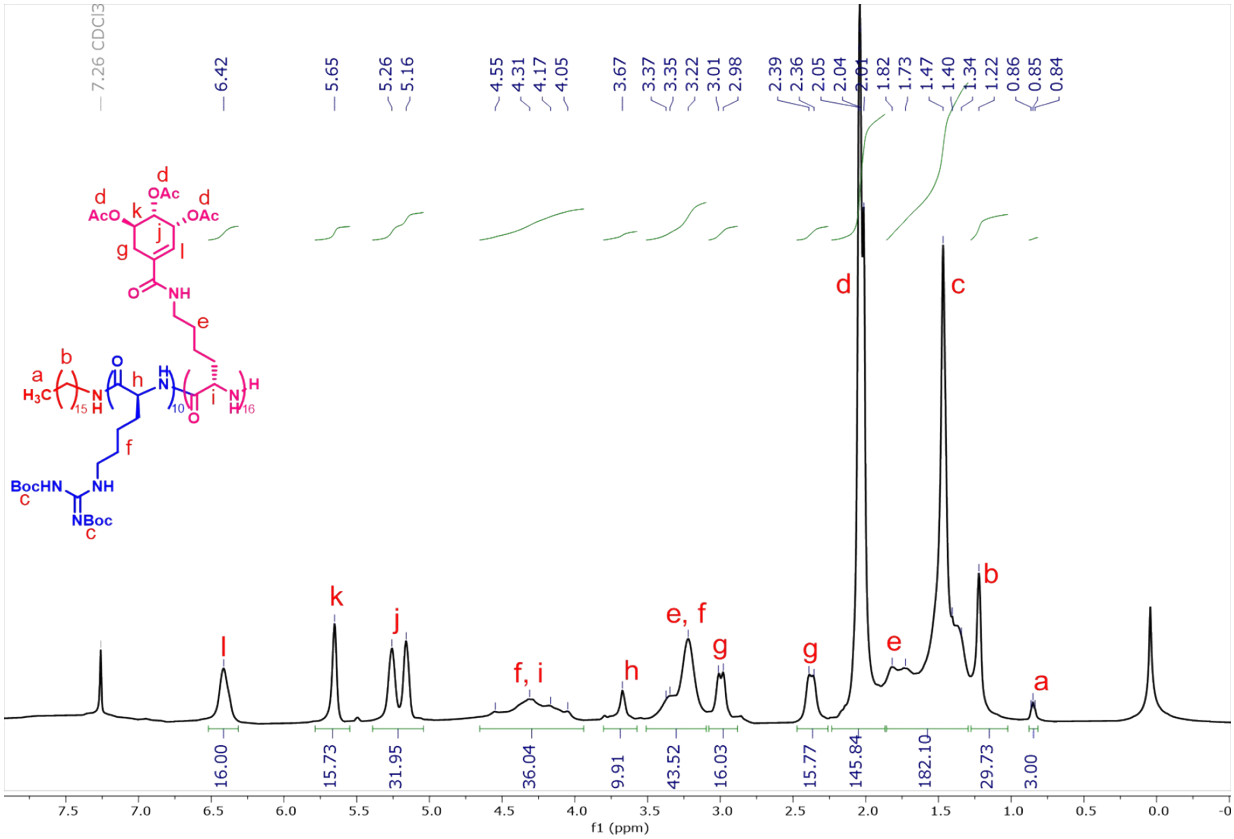


Figure S39: ^{1}H NMR of fully protected HDA-*b*-PHAG$_{10}$-*b*-PLSA$_{16}$

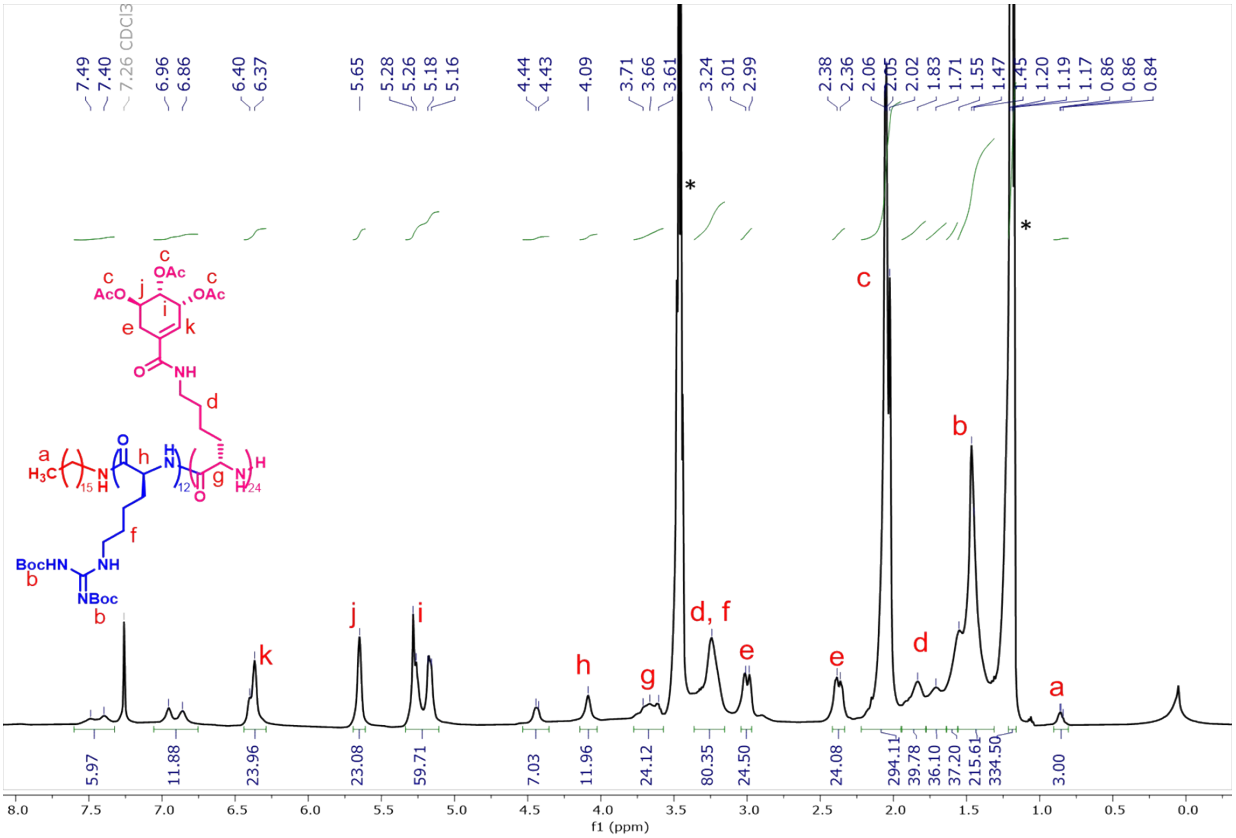


Figure S40: ^{1}H NMR of fully protected HDA-*b*-PHAG$_{12}$-*b*-PLSA$_{24}$

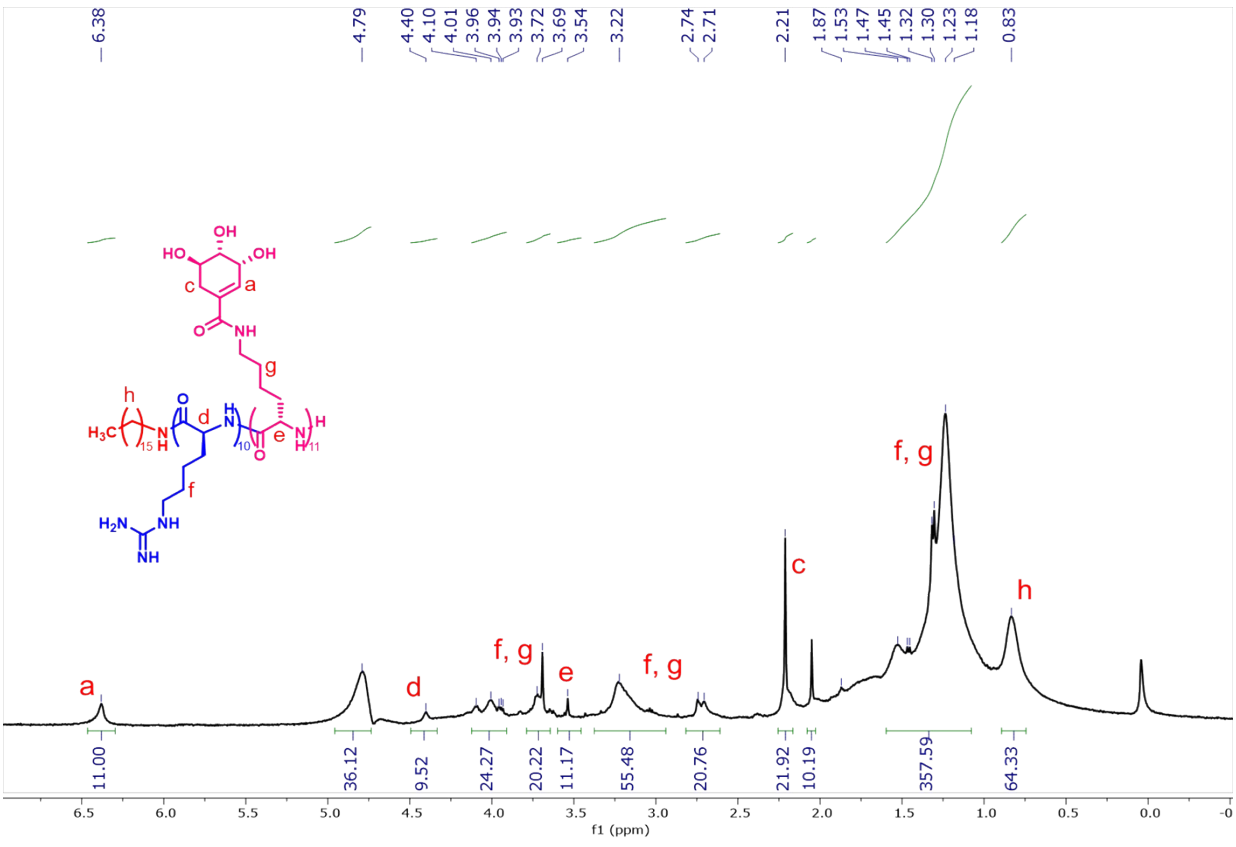


Figure S41: ^{1}H NMR of fully deprotected HDA-*b*-PHAG$_{10}$-*b*-PLSA$_{11}$

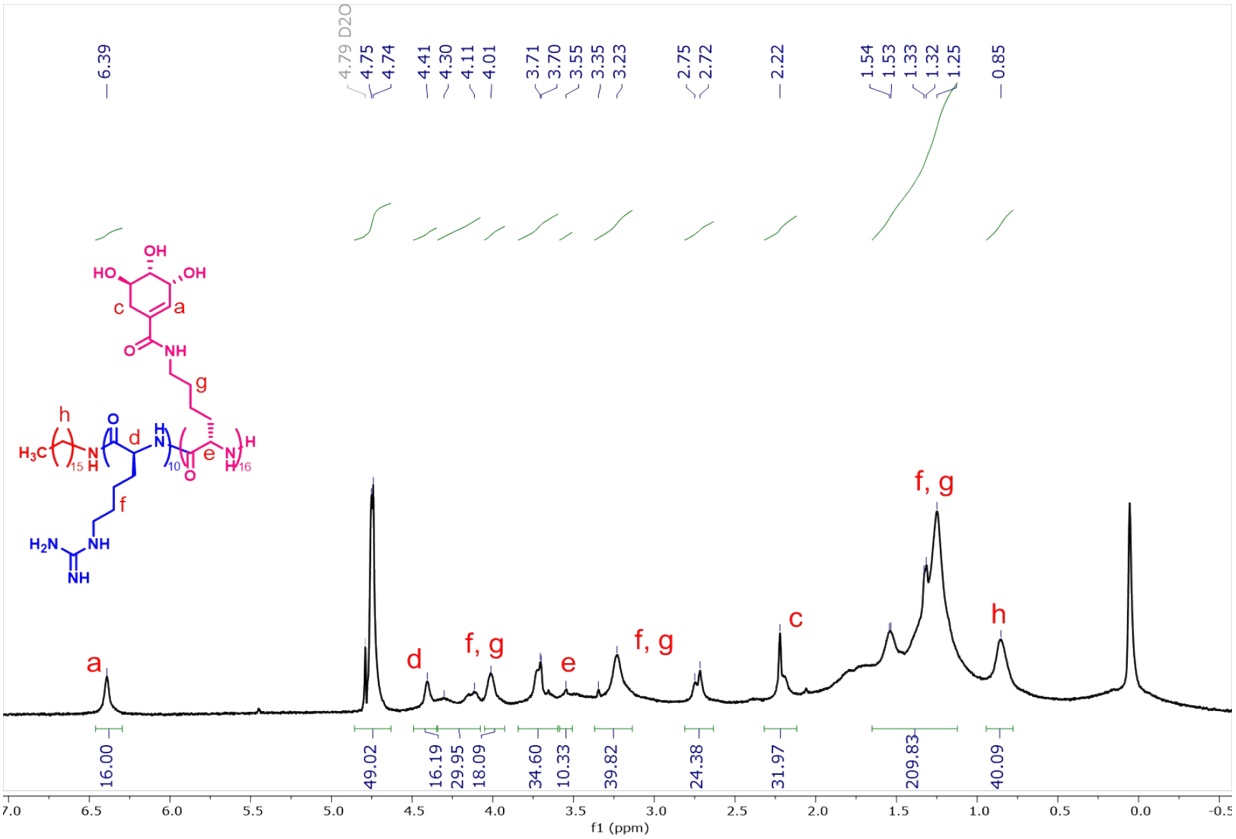


Figure S42: ^{1}H NMR of fully deprotected HDA-*b*-PHAG$_{10}$-*b*-PLSA$_{16}$

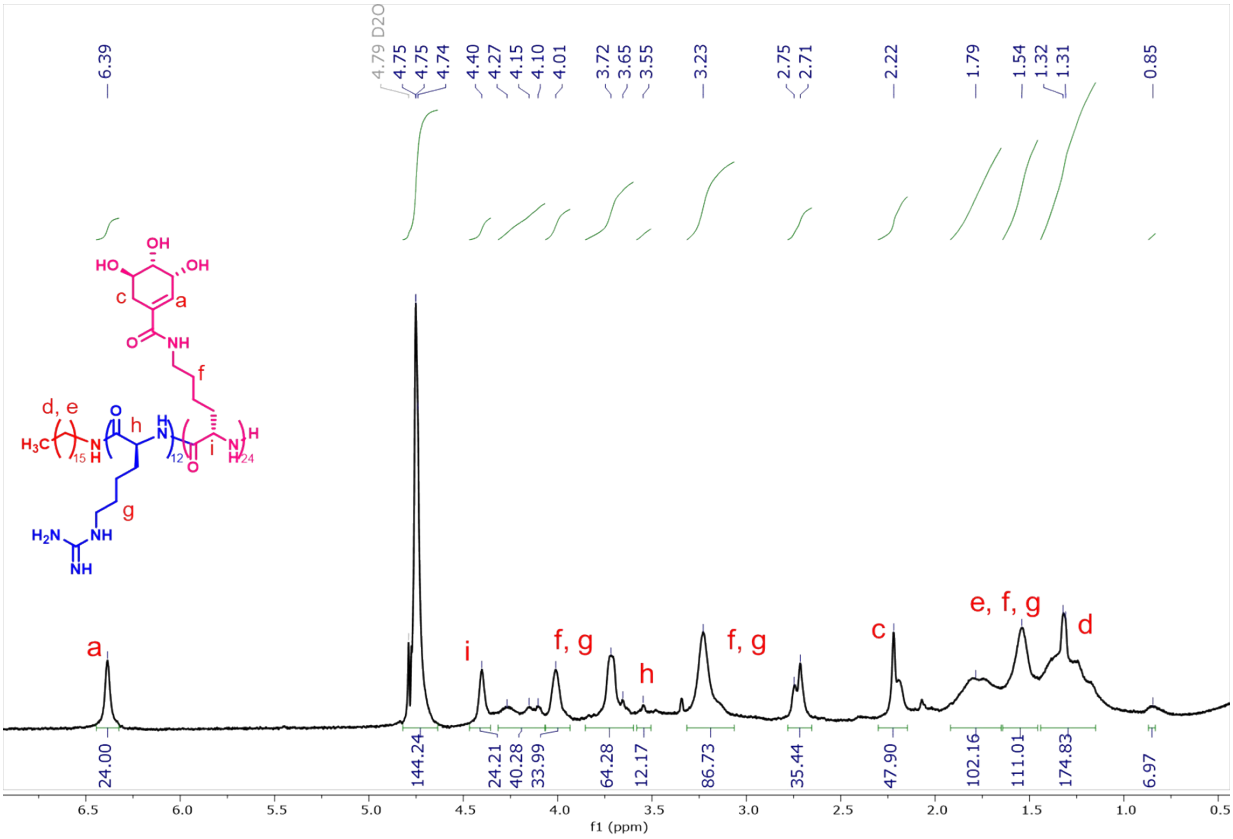


Figure S43: ^{1}H NMR of fully deprotected HDA-*b*-PHAG$_{12}$-*b*-PLSA$_{24}$

1. K. Praveen, S. Das, V. Dhaware, B. Pandey, B. Mondal and S. S. Gupta, *ACS Applied Bio Materials*, 2019, **2**, 4162-4172.