Intracellular Delivery of Virus-Like Particles Using a Sheddable Linker

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Supporting Information

Materials and Methods

(3,4-dibromomaleimide, N-methylmorpholine, methylchloroformate, Di-tert-butyl dicarbonate ($(Boc)_2O$), ethylenediamine, 6-bromohexanoic acid, triphenylphosphine, N-hydroxysuccinimide (NHS), and triethylamine were purchased from Sigma-Aldrich, Alfa Aesar, and Chem Impex. Potassium chloride, FBEssence, potassium phosphate monobasic, potassium phosphate dibasic, Dulbecco's Modified Eagle's Medium (6429), Tris base, sodium phosphate monobasic, tryptone, sodium phosphate dibasic, yeast extract, sodium dodecyl sulfate, sodium chloride, peptone, glycine, and Coomassie Brilliant Blue reagent were used without further purification. Reagents were purchased from Research Product International (Mt Prospect, IL, USA), VWR (Radnor, PA, USA), Chem-Impex Int'l (Wood Dale, IL, USA), Thermo Fisher Scientific (Waltham, MA, USA), and Sigma-Aldrich (St. Louis, MO, USA). siRNA HPLC purified siRNA luciferase(5'-GAUUAUGUCCGGUUAUGUA[dT][dT]-3'antisense:3' UACAUAACCGGACAUAAUC[dT][dT]-5') was purchased from Sigma Aldrich.

Size exclusion chromatography. Size exclusion chromatography was accomplished on an Agilent 1100 series HPLC system on a GS400SWXL (7.8 mm × 300 cm) column (flow rate 0.5 mL/min) using an aqueous mobile phase (0.1M Sodium Phosphate buffer, pH 7.4).

Gel analyses. 10% Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was conducted on a BioRad Mini-PROTEAN Tetra Cell system. All electrophoresis protein samples were mixed with SDS loading buffer and heated to 95 °C for 5 min to ensure complete denaturation. Molecular mass markers from Fisher Scientific were applied to approximate the apparent molecular masses. Gels were run at 200V for 60 mins using SDS running buffer. Gels were imaged using a BioRad ChemiDoc Touch Imaging System. 1% agarose gels were used throughout the experiments. Experimental parameters for running the gel include the following: 100 V, 30 mins, and a running solution consisting of 1× TBE buffer. Samples were prepared in 50% glycerol.

Fast protein liquid chromatography (FPLC). VLP purification was performed using a BIO-RAD NGC Chromatography System. We used a size-exclusion Superose-6 Increase 10/300 GL column for all purification purposes. All trials used potassium phosphate buffer (1 M, pH 7.4) as the eluting solution, a flow rate of 0.3 mL/min, and sample collection was done at RT. All particles eluted between 12–14 mL.

Transmission electron microscopy (TEM). Transmission electron micrographs were taken on a JEOL JEM-1400+ (JEOL, Tokyo, Japan) at 120 kV with a Gatan 4k × 4k CCD camera. 5 µL of the ~0.1 mg/mL desalted sample was placed on a 300 mesh Formvar/carbon-coated copper grid (Electron Microscopy Sciences, Hatfield, PA, USA), allowed to stand for 30 seconds, and wicked off with Whatman #1 filter paper. 5 µL of 2% uranyl acetate (SPI Supplies, West Chester, PA, USA) was placed on the grid, allowed to stand for 30 seconds, wicked off as before, and the grid was allowed to dry completely in air.

Dynamic-light scattering. Analysis was done with a UV-Vis Malvern Panalytical Zetasizer Nano ZS. A disposable microcuvette was used throughout the measurements. Experimental parameters included the following: 25 °C, a 175 ° scattering angle, a medium refractive index of 1.33, a 633 nm laser, and a material refractive index of 1.51.

Centrifugations and purification*:* Centrifugations were conducted using a Fiberlite F10 rotor at 19,510 ×g and a Sorvall Legend Micro 17 tabletop centrifuge at 17,000 ×g. General desalting and removal of other small molecules of biological samples were achieved using Amicon Ultra-15, either 10 KDa or 100 kDa molecular weight cut-off (MWCO) centrifugal filter units. Dialysis was performed using Fisherbrand™ Regenerated Cellulose Dialysis with a 3.5 kDa MWCO.

Bradford assay: The amount of Qβ before and after bioconjugation was measured using the Bradford method with bovine serum albumin as the standard. All protein determination values were based on the average of triplicate measurements.

Expression and purification of Qβ and Qβ(GFP). The expression and purification of Qβ VLPs were done using a published procedure, which is reproduced here in brief.[1] The plasmids were gifts from Prof. M.G. Finn of the Georgia Institute of Technology. In a 10 mL of starter culture of E. coli BL21 cells with the plasmid were amplified to 500 mL of SOB media (100 µg/mL kanamycin) at 37 °C until the OD600 was 0.9-1.0. To that, 1 mM (final concentration) of isopropyl β-D-1-thiogalactopyranoside (IPTG) was added to induce the expression at 37 °C overnight. Cells were harvested by centrifuging using a Fiberlite F10 rotor at 10,500 rpm (19,510 × g) for one hour at 4 °C, followed by re-suspending into 70 mL of 0.1 M potassium phosphate buffer (pH 7.00). Cells were lysed in a cell homogenizer, and the lysate was centrifuged in a Fiberlite F10 rotor at 10,500 rpm (19,510 × g) for 1 h at 4 °C. The pellet was discarded, ammonium sulfate was added to the supernatant to produce a final concentration of 2 mM, and the solution was incubated with rotation for at least 1 h at 4 °C. The suspension was centrifuged using a Fiberlite F10 rotor at 10,500 rpm (19,510 × g) for one hour at 4 °C. The pellet was re-suspended in 10 mL of 0.1 potassium phosphate buffer (pH 7.00), and an equal volume solution of n-butanol and chloroform (1:1) was added. The solution was vortexed and pelleted by centrifuge in a Fiberlite F10 rotor at 10,500 rpm (19,510 × g) for 30 min at 4 °C. The top aqueous layer was carefully recovered and further purified by 10-40% sucrose gradient in a SW-28 rotor at 23,500 rpm (73,078 × g) for 16 hours at 4 °C. The band containing Qβ particles was visualized by white light—typically from the flash of a cell phone—from the bottom of the tube and extracted using a long needle. This extract was pelleted using a Ti-70 rotor at 60,000 rpm (264,902 ×g) for 2.5 hours. The pellet was re-suspended into the desired buffer.

Bioconjugation of TPP-DB to Qβ. Qβ solution was prepared in a 10 mM sodium phosphate solution (pH 5.00) and the disulfide bonds were reduced by addition of tris(2-carboxyethyl) phosphine (TCEP) (10 eq per disulfide) at RT for one hour (each Qβ has 180 disulfide bonds). 20 eq of TPP-DB compound dissolved in 10% DMF was added to the reduced Qβ solution (1 mg/mL). The

reaction was incubated at RT overnight. The conjugated product was purified by centrifuge filter (4300 × g, 20 mins × 3, 10,000 MWCO) using 10 mM sodium phosphate solution (pH 5.00).

Bioconjugation of TPP-NHS to Qβ. Qβ-TPP conjugates are not soluble and precipitate in phosphate buffer, so the reaction was done in HEPES buffer. TPP-NHS ester in DMF (20 eq) was added to Qβ in HEPES and incubated at room temperature for 6 h on a rotisserie. The conjugates were purified by centrifugal filter (4300 ×g, 20 mins ×3, 10,000 MWCO) or PD10 desalting column to get rid of excess small molecules using HEPES.

Bioconjugation of DB-PEG to Qβ. Qβ was prepared in 10 mM sodium phosphate solution (pH 5.00) and first reduced with 10 equivalents of TCEP, followed by the addition of 50 eq dibromomaleimide-PEG (DB-PEG). The reaction was then left on a rotisserie overnight at RT before purifying using a centrifugal filter (4300 ×g, 20 mins ×3, 10,000 MWCO) or a PD10 desalting column with HEPES buffer. Conjugation of TPP-NHS with surface amines was then performed using the same method as bioconjugation of TPP-NHS to Qβ.

Cytoplasm mimicking cleavage experiment. To evaluate the cytoplasm GSH-triggered release mechanism of the maleimide linker, we first labeled dibromaleimide with Fluorescein Isothiocyanate (FITC). To investigate the cleavage behavior of QB-M-FITC using an artificial cytoplasm environment, a solution of Qβ-M-FITC (2 mg/ml) was incubated in a solution of 20 mM HEPES, 100 mM KCl, 1 mM $MgCl_2$, 1 mM EDTA, pH 7.4 and 1 mM glutathione, which mimics cytoplasm conditions. The mixture was vortexed for 10 s, then incubated at 37 °C for 24 h, and the DB-FITC release was monitored by size exclusion chromatography over 24 h.

Cytotoxicity of Qβ(GFP)-DB-TPP. To investigate the cytotoxicity of Qβ(GFP)-DB-TPP on A549 cells, 100 µL of 2 mg/ml of the conjugated of Qβ(GFP)-DB-TPP and Qβ(GFP) was incubated in a 96 well plate with 10^5 cells for 4 h, and the viability was compared to control cells by a standard colorimetric 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Cellular viability was determined based on the conversion of the water-soluble yellow MTT dye to an insoluble purple formazan over 2 h. Formazan was then solubilized with 100 µL of DMSO before reading the absorbance of each well at 570 nm (n = 3).

Uptake of Qβ(GFP)-DB-TPP. To measure the uptake of Qβ(GFP)-DB-TPP on A549 cells, 500 µL of 2 mg/ml of the conjugated Qβ(GFP)-DB-TPP and Qβ(GFP) was incubated in a 24 well plate with 10^6 cells for 4 h. The cells were trypsinized with 1× trypsin-EDTA, moved to Eppendorf tubes, washed 3× with 1× PBS, fixed with 4% PFA for 15 mins at RT, washed 3× with 1× PBS, and transferred into cell culture tubes for analysis by flow cytometry. GFP fluorescence was measured on a BD Bioscience LSRFortessa, and the fluorescence intensity value of Qβ(GFP)-DB-TPP and Qβ(GFP) were compared using FlowJo v10 software.

Cytosolic delivery of Qβ(GFP)-M-TPP. To study the *in vitro* GSH sensitive behavior of Qβ(GFP)-DB-TPP, A549 cancer cells (10^5 cells) were seeded one night before the experiment in a 24 well plate in serum-free media DMEM, supplemented with 1% Pen-Strep cell culture media, and incubated at 37 °C, 5% CO_2. Cells were treated with either Qβ(GFP) and Qβ(GFP)-DB-TPP in 2 mg/ml concentration for 2 h before being washed 3× with serum-free media and 3× with PBS. The cells were stained with DAPI for nucleus staining or lysotracker blue and imaged by epifluorescence microscopy.

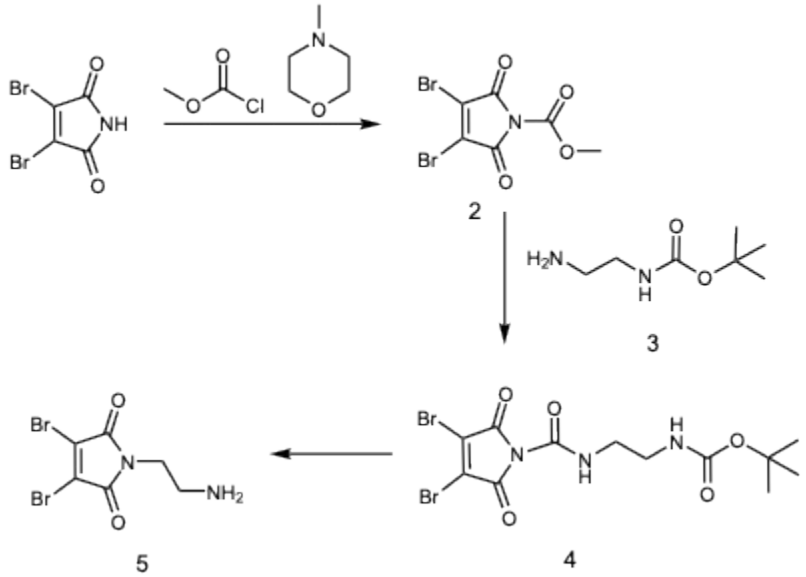
Disassembly of Qβ Bacteriophages. To harvest coat proteins (CPs), 45 mg Qβ (2.5 mg/mL) in PBS was disassembled directly into monomers under denaturing conditions when treated with 10 mM DTT for 15 min at RT. The solution was then centrifuged at

650 rpm (87 ×g) for 10 min and treated with 0.7 M $MgCl_2$ for 1h to precipitate the RNA. The disassembled CP/RNA mixture was then centrifuged at 4,000 rpm (3,283 xg) (Allegra X-14R) for 10 min at 4 °C, and the supernatant was collected for purification using a 100,000 MWCO spin column to remove capsids from CPs.

Encapsulation of siRNA inside Qβ capsid. To assemble Qβ around siRNA, we modified a procedure reported by the Bachmann group.[2] Coat proteins (1 mg/ml) were mixed with 0.24 mg/ml siRNA in the presence of 1.0 M urea, 250 mM NaCl, and 2.5 mM DTT. The mixture was dialyzed at RT with 300 mL of 20 mM sodium phosphate and 250 mM NaCl (pH 7.2) using a 3.5 kDa cut-off cartridge. After 4 h of dialysis, DB-TPP (50 eq to CP) was added to the mixture and allowed to react for 1 h at RT, followed by dialysis with a 300 kDa cut-off cartridge against 500 mL of PBS.

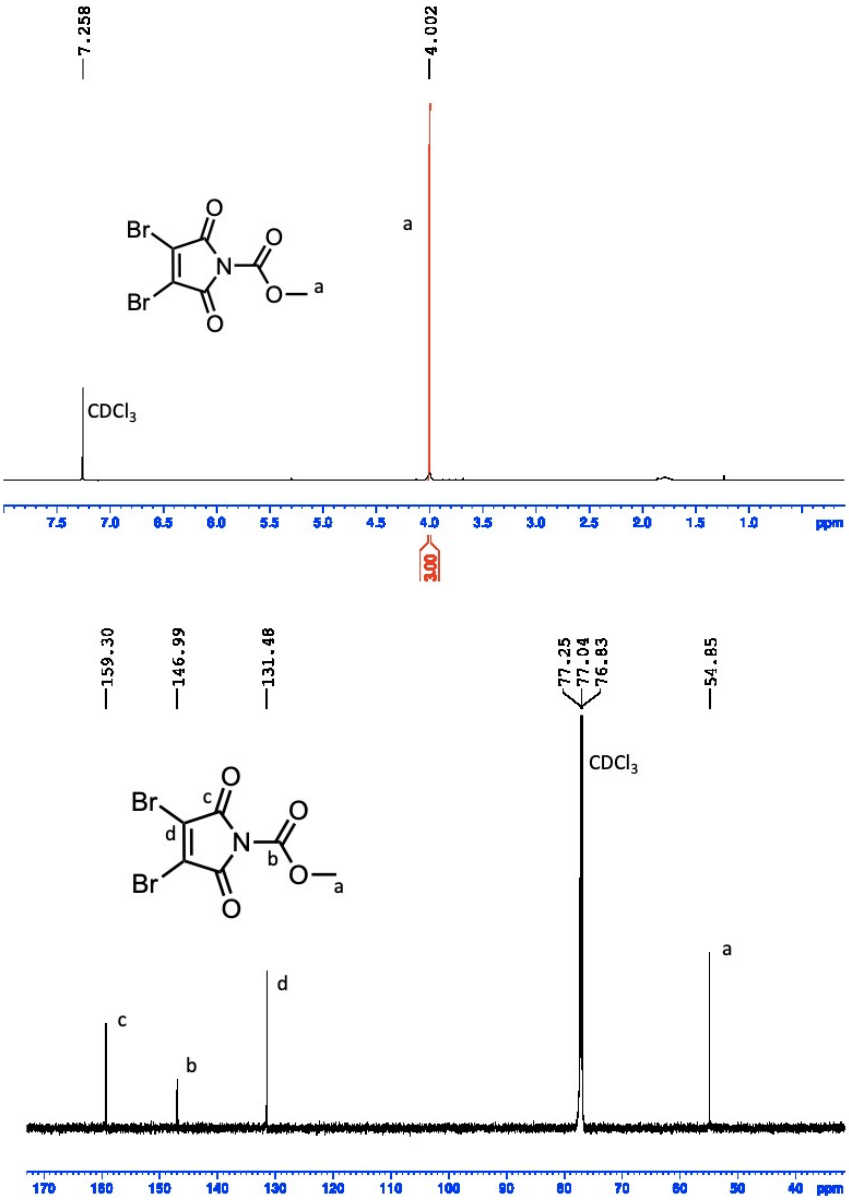
Evaluation of in vitro RNA delivery using One Glo +Tox assay: HeLa cells expressing firefly luciferase (HeLa-Luc) were seeded (10,000 cells/well in an opaque white 96-well plate) a day before the experiment and allowed to attach to the plate overnight in phenol red-free Dulbecco's modified Eagle's medium (DMEM). Then 100µl of 2mg/ml Qβ-M-TPP encapsulated anti-luciferase siRNA, naked siRNA, Qβ-M without TPP moiety encapsulated anti-luciferase siRNA, and Qβ-M-TPP was added to each well and incubated for 24 h. Firefly luciferase activity and viability were analyzed using One Glo + Tox assay kits. This assay is a two-step procedure with an "add-mix-read" format that checks luciferase gene expression in viable cells. The first step is checking viability based on an AFC fluorophore that measures live-cell-protease activity. To test this, 20µl of CellTiter-Fluor™ Reagent (prepared as 5× solution using 10µl of GF-AFC Substrate in 2ml of Assay Buffer) was added to all wells, and mixed briefly with an orbital shaker at 500 rpm and incubated for 1 h at 37 °C. Then fluorescence was read using a fluorometer at 380–400nmEx/505nmEm. The second part of the assay uses the ONE-Glo™ Luciferase Assay system to quantify the firefly luciferase reporter gene expression by measuring luminescence. For this purpose, 100µl of ONE-Glo™ Reagent was added to each well and incubated for 3 minutes, and then luminescence was measured using a luminometer.

Synthesis of DB-amine



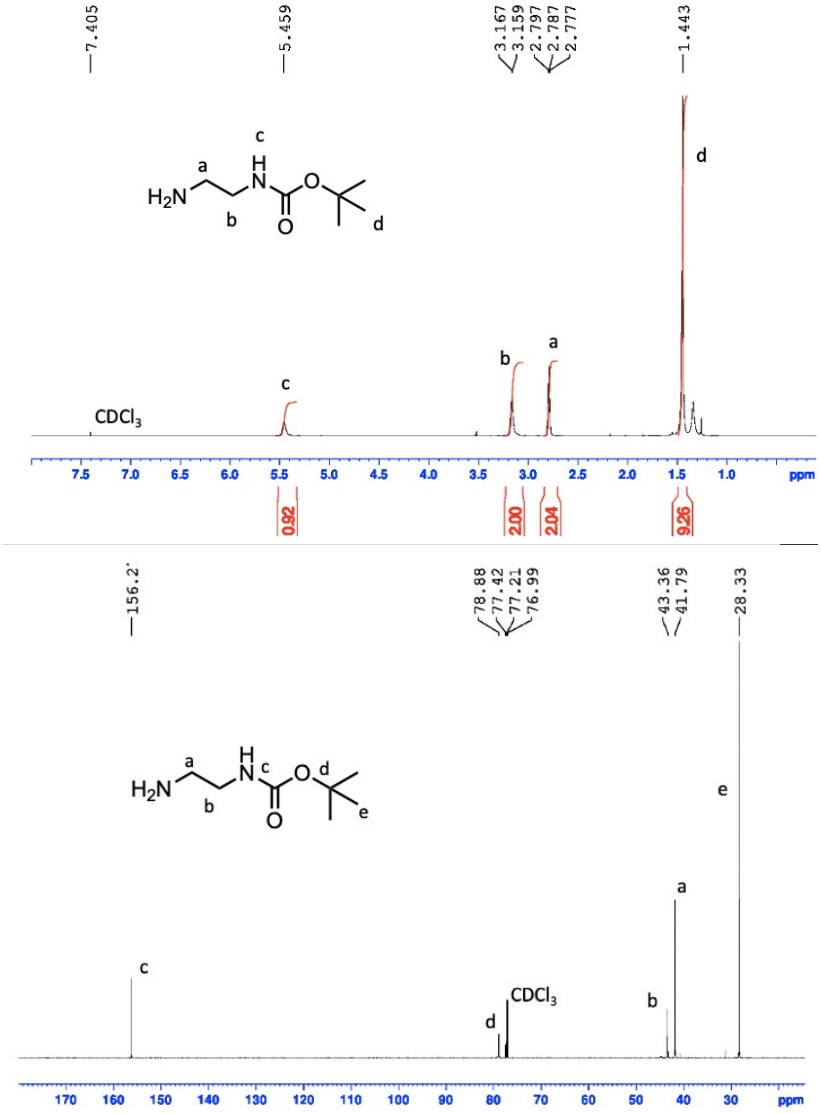
Synthetic pathway of Dibromomaleimide-triphenyl phosphonium linker.

Synthesis of Methyl 3,4-dibromo-2,5-dioxo-2,5-dihydro-1H-pyrrole-1-carboxylate (Compound 2): 3,4-dibromomaleimide (1.0 g, 3.9 mmol) and N-methylmorpholine (0.43 ml, 3.9 mmol) were dissolved in 35 mL of THF and methylchloroformate (0.30 ml, 3.9 mmol) was added to the mixture. The reaction was stirred at room temperature (RT) for 20 min. After stirring, 40 mL of dichloromethane (DCM) was added, and the organic phase was washed with water (liquid-liquid extraction). The organic phase was dried over anhydrous $MgSO_4$, filtered, and the solvent was evaporated under reduced pressure to yield a pink solid. Yield: (1.15g, 3.70 mmol, 94%) ^{1}H NMR (600 MHz, $CDCl_3$) δ ppm 4.00 (s, 3 H). ^{13}C NMR (600 MHz, $CDCl_3$) δ ppm 54.85, 131.48, 146.99, 159.30.



^{1}H and ^{13}C NMR of compound 2.

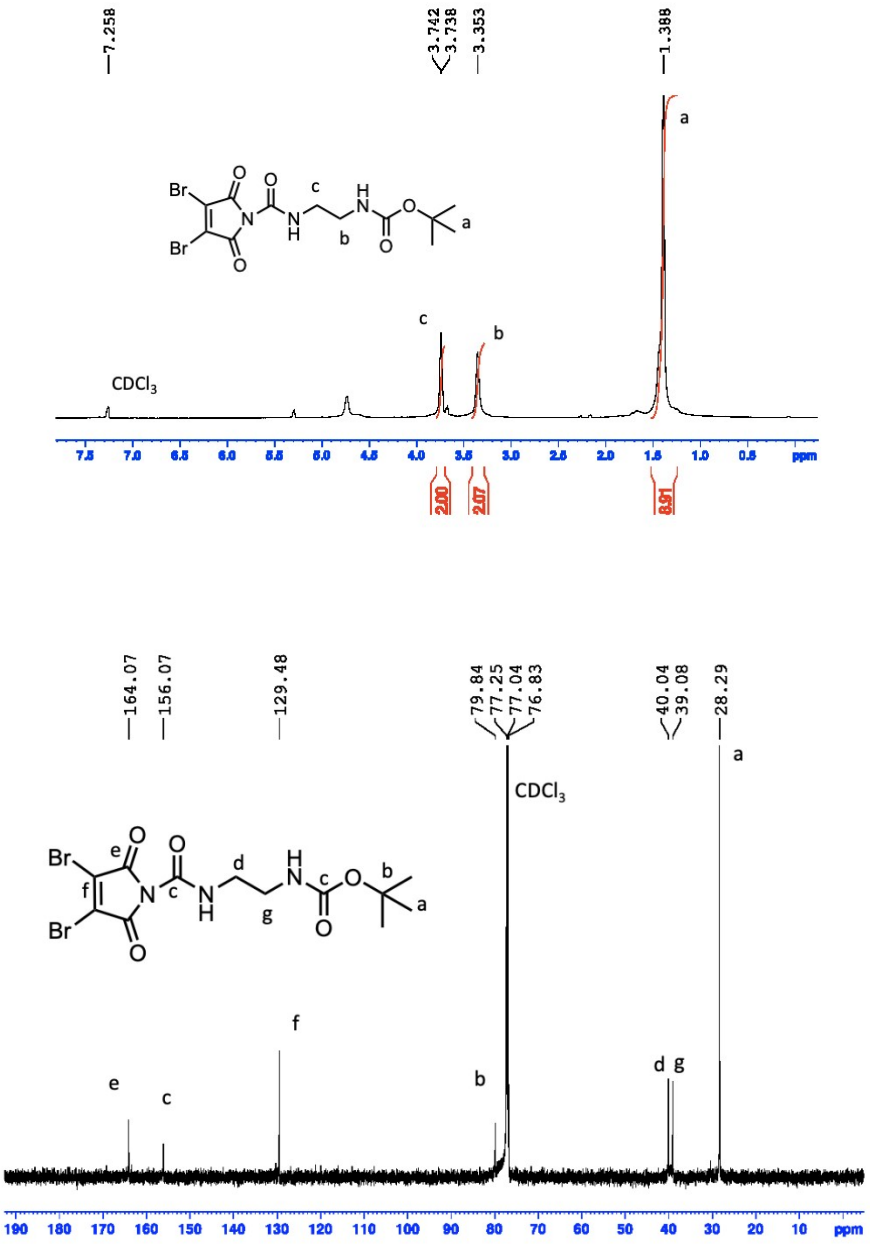
Synthesis of tert-butyl N-(2-aminoethyl) carbamate (Compound 3): To a solution of $(Boc)_2O$ (5.0 g, 23 mmol) dissolved in DCM (10 mL) was added a solution of ethylenediamine (30.63 ml, 458.2 mmol) in DCM (50 mL) at 0°C. After addition, the mixture was allowed to reach RT and stirred for 2 h. The organic phase was washed with water and dried over anhydrous $MgSO_4$. The organic layer was separated and concentrated under reduced pressure. The residue was again dissolved in 20 mL of diethyl ether and 20 mL of brine. The mixture was acidified to a pH of 5.00 using a 4 M solution of HCl. The di-protected ethylenediamine was extracted in diethyl ether and discarded. The pH of the aqueous phase was again adjusted to 10.50 with a 2 M solution of NaOH and extracted with ethyl acetate. The organic phase was dried over anhydrous $MgSO_4$, filtered, and the solvent was evaporated under reduced pressure to yield the product as a yellow oil (2.24g, 14.0 mmol, 60%). ^1H NMR (600 MHz, $CDCl_3$) δ ppm 1.44 (9H, s), 2.79 (2H, t, J=5.89 Hz), 3.16 (2H, d, J=5.18 Hz), 5.46 (1H, bs). ^{13}C NMR (600 MHz, $CDCl_3$) δ ppm 28.33, 41.79, 43.36, 78.88, 156.27.



^1H and ^{13}C NMR of compound 3.

Synthesis of tert-butyl N-[2-(3,4-dibromo-2,5-dioxo-2,5-dihydro-1H-pyrrol-1-y) ethyl] carbamate (Compound 4): To synthesize compound 4, compound 2 (1.050 g, 3.360 mmol) and compound 3 (0.5400 g, 3.360 mmol) were each dissolved in DCM (10 mL).

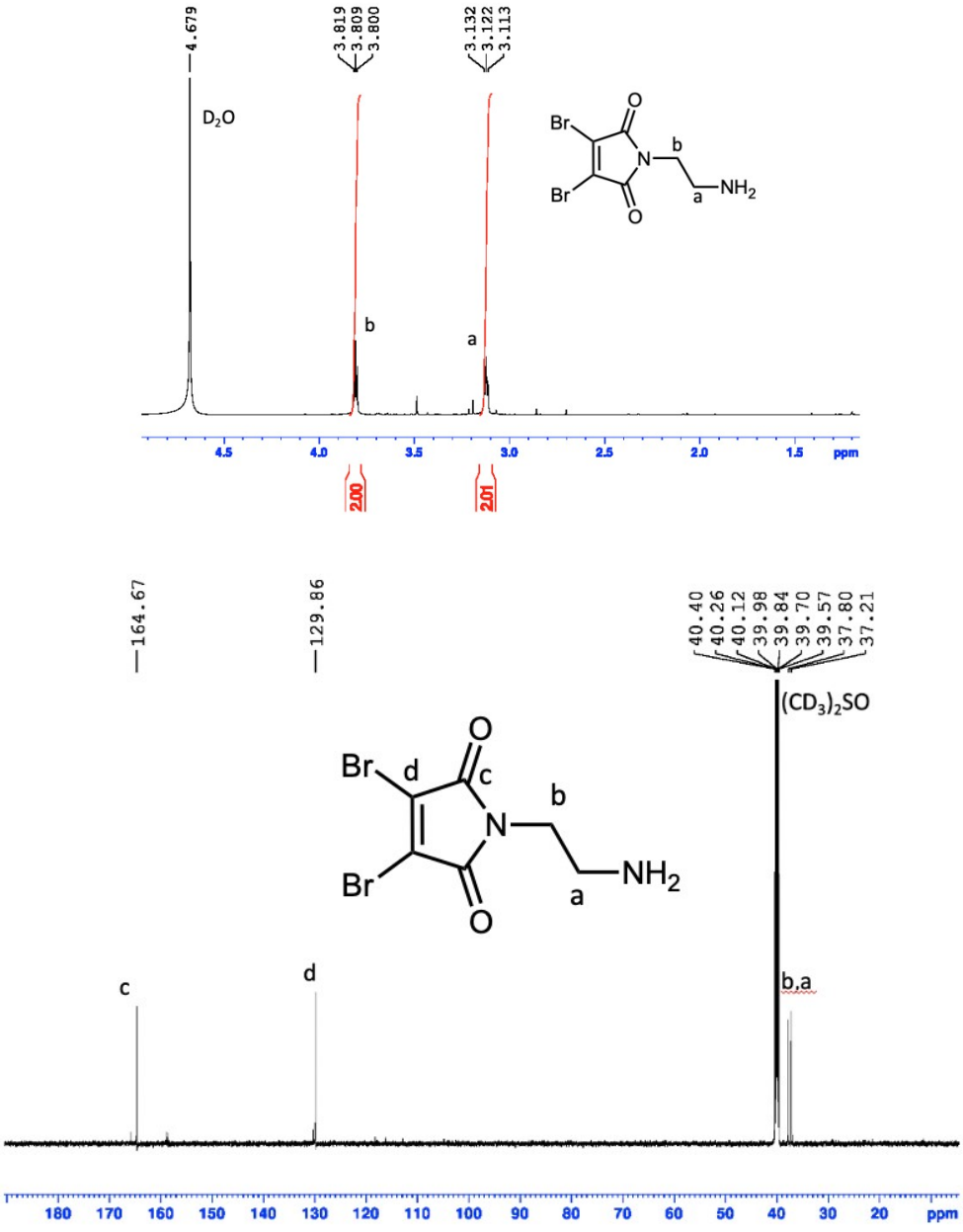
The compound 3 solution was slowly added to the compound 2 solution and allowed to stir for 2 h. The reaction was then washed, and the organic phase was separated, dried with anhydrous $MgSO_4$, filtered, and then removed under reduced pressure. (0.60 g, 1.4 mmol, 51%) ^{1}H NMR (600 MHz, $CDCl_3$) δ ppm 1.39 (9H, s), 3.35 (2H, d), 3.74 (2H, d, J=2.27 Hz). ^{13}C NMR (600 MHz, $CDCl_3$) δ ppm 28.29. 39.08, 40.04, 79.84, 129.48, 156.07, 164.07.



^{1}H and ^{13}C NMR of compound 4.

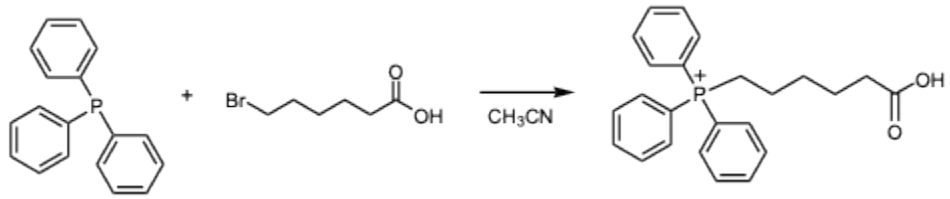
Synthesis of 2-(3,4-dibromo-2,5-dioxo-2,5-dihydro-1H-pyrrol-1-yl) ethan-1-aminium trifluoroacetate (Compound 5):

To remove the Boc protecting group, compound 4 (1.0 g, 2.5 mmol) was dissolved in 20 mL of DCM/trifluoroacetic acid (TFA) (1:1), and the solution was stirred at RT for 1 h. The solvent was evaporated under reduced pressure to yield white crystals (0.90 g, 3.1 mmol, 81%). ^{1}H NMR (600 MHz, D_2O) δ ppm 3.12 (2H, t, J=5.79 Hz), 3.81 (2H, t, J=5.83 Hz). ^{13}C NMR (600 MHz, $(CD_3)_2SO$) δ ppm 37.21, 37.80, 129.86, 164.67.

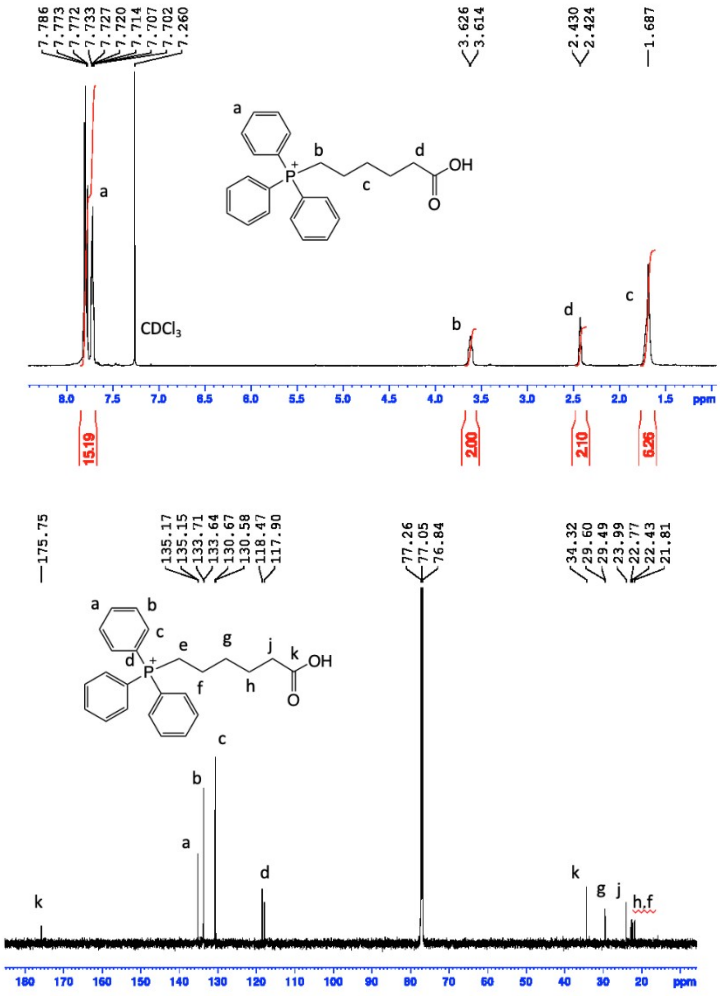


^{1}H and ^{13}C NMR of compound 5.

Synthesis of (5-carboxypentyl)triphenylphosphonium (TPP-acid):

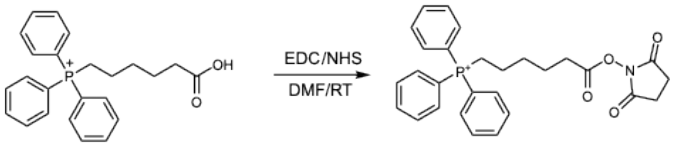


A solution of 6-bromohexanoic acid (8.0 g, 41 mmol) and triphenylphosphine (11.3 g, 43.2 mmol) in 50 mL acetonitrile was refluxed under N_2 nitrogen for 16 h. Upon cooling to room temperature, the product began to crystallize. The crystal product was filtered and washed with Et_2O (4 ×30 mL) to afford a white powder (14.4 g, 38.1 mmol, 93%). ^{1}H NMR (600 MHz, $CDCl_3$) δ ppm 1.69 (6H, s), 2.43 (2H, t, J=3.15 Hz), 3.62 (2H, t, J=6.74 Hz), 7.75 (15H, m, J=6.29 Hz). ^{13}CNMR (600 MHz, $CDCl_3$) δ ppm 21.81, 22.43, 22.77, 23.99, 29.49, 29.60, 34.32, 117.90, 118.47, 130.58, 130.67, 133.64, 133.71, 135.15, 135.17, 175.75

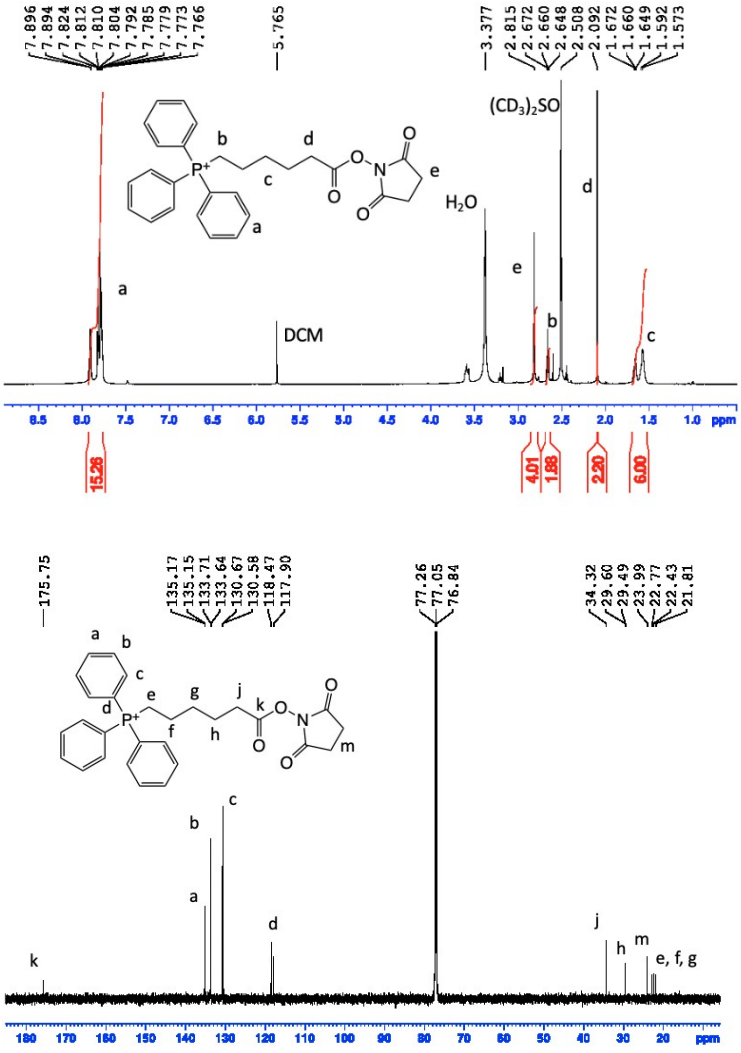


^{1}H and ^{13}C NMR of TPP-acid.

Synthesis of (6-((2,5-dioxopyrrolidin-1-yl) oxy)-6-oxohexyl) triphenylphosphonium (TPP-NHS):

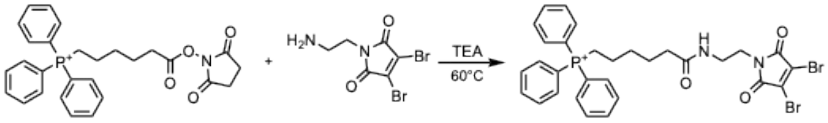


To a flask with (5-carboxypentyl) triphenylphosphonium (0.64g, 1.4 mmol dissolved in 10 ml of DMF was added 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) (0.60 g, 3.8 mmol) and N-hydrosuccinimide (NHS) (0.49 g, 4.2 mmol). The reaction was stirred at RT overnight. the solvent was evaporated under reduced pressure and the residue was further purified by silica chromatography (gradient 0–20% MeOH in DCM) to yield a white solid (0.25 g, 0.10 mmol, 37%). ^{1}H NMR (600 MHz, DMSO) δ ppm 1.64 (6H, m, J=14.90 Hz), 2.09 (2H, s), 2.66 (2H, t, J=7.26 Hz), 2.82 (4H, d), 7.84 (15H, m, J=7.13 Hz). ^{13}C NMR (600 MHz, DMSO) 21.81, 22.43, 22.77, 23.99, 29.49, 34.32, 117.90, 118.47, 130.58, 130.67, 133.64, 133.71, 135.15, 135.17, 175.75

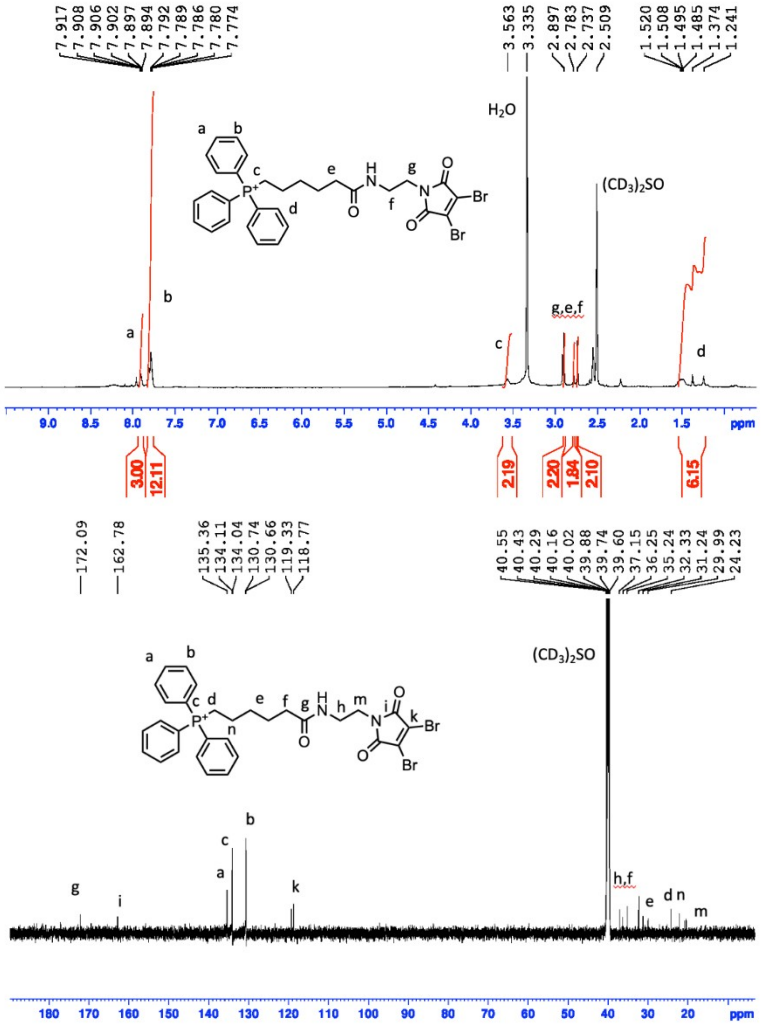


^{1}H and ^{13}C NMR of TPP-NHS.

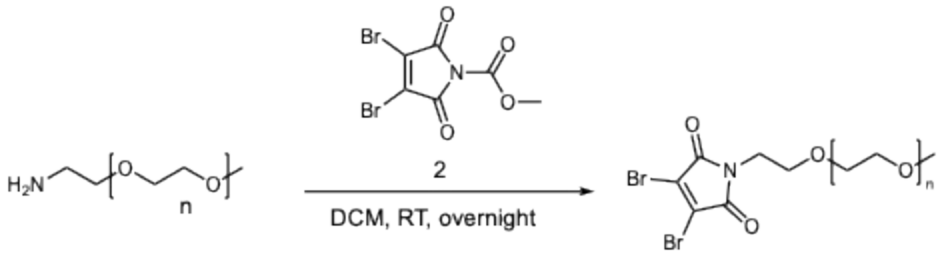
Synthesis of (6-((2-(3,4-dibromo-2,5-dioxo-2,5-dihydro-1H-pyrrol-1-yl) ethyl) amino)-6-oxohexyl)triphenylphosphonium. (TPP-DB):



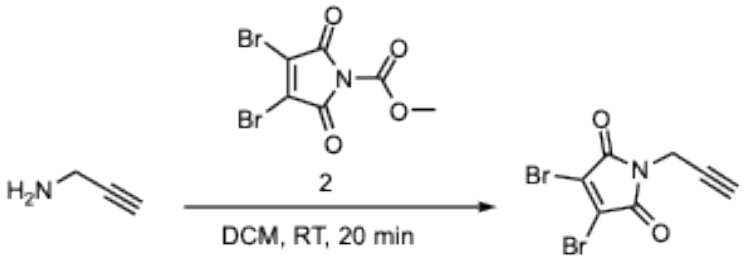
Compound 5 (0.18 g, 0.44 mmol) was dissolved in DMF (10mL), and triethylamine (TEA) (0.06 mL) was added to solution and stirred for 0.5 h. TPP-NHS (0.23 g, 0.49 mmol) was dissolved in DMF (5 mL) and added to the stirred solution of compound 5 at 60 °C for 72h. The reaction mixture was cooled down to RT, solvent was removed, and crude were purified with basic alumina column under gradient of 0–10% MeOH:DCM. The solvent was removed and the product was collected (0.116 g, 0.177 mmol, 37%). ^{1}H NMR (600 MHz, DMSO) δ ppm 1.45 (6H, m, J=21.89 Hz), 2.73 (2H, t, J=3.86 Hz), 2.78 (2H, t), 2.90 (2H, t, J=3.54 Hz), 3.56 (2H, t), 7.87 (15H, m, J=10.10 Hz), 8.22 (1H, bs). ^{13}C NMR (600 MHz, DMSO) δ ppm 24.23, 29.99, 31.24, 32.33, 35.24, 36.25, 37.15, 118.77, 119.33, 130. 66, 130.74, 134.04, 134.11, 135.36, 162.78, 172.09. ESI) *m/z*: [M -Br] calculated for $C_{30}H_{30}Br_2N_2O_3P^+$ 657.40; found, 657.14.



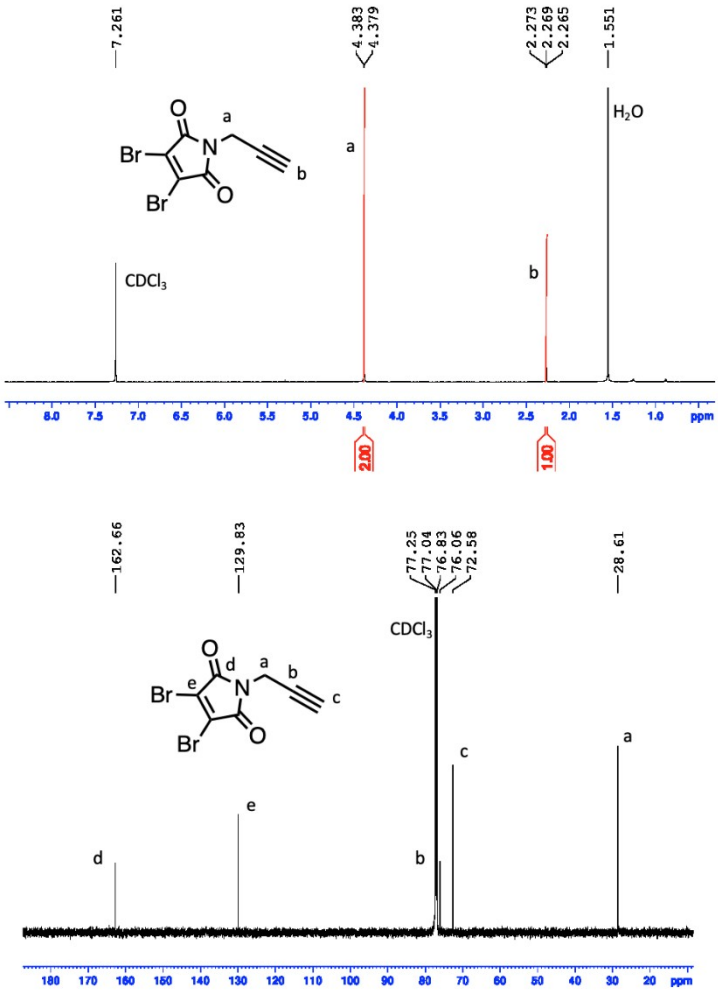
^{1}H and ^{13}C NMR of TPP-DB.



Synthesis of dibromomaleimide-PEG: To a solution of compound **2** (0.1 g, 0.4 mmol) in 50 mL of DCM, methoxypolyethylene glycol amine 1000 (0.5 g, 0.5 mmol) was added, and the reaction mixture was stirred overnight. The solvent was removed under reduced pressure and purified using silica chromatography (gradient 0-30% MeOH in DCM) to yield a yellow oil (0.4 g, 0.3 mmol, 64%). ^{1}H NMR (500 MHz, $CDCl_3$) δ ppm 3.31 (s, 3 H) 3.47-3.49 (t, J=5.00, 2H) 3.50 - 3.62 (m, 88 H) 3.75 (t, J=5.00, 2H). ^{13}C NMR (126 MHz, $CDCl_3$) δ ppm 38.94, 52.19, 59.05, 67.54, 70.08, 70.58, 71.94, 129.43, 163.81. HRMS: Mass calculated for $[M+H]^+$ 1236.4376, observed 1236.4414.

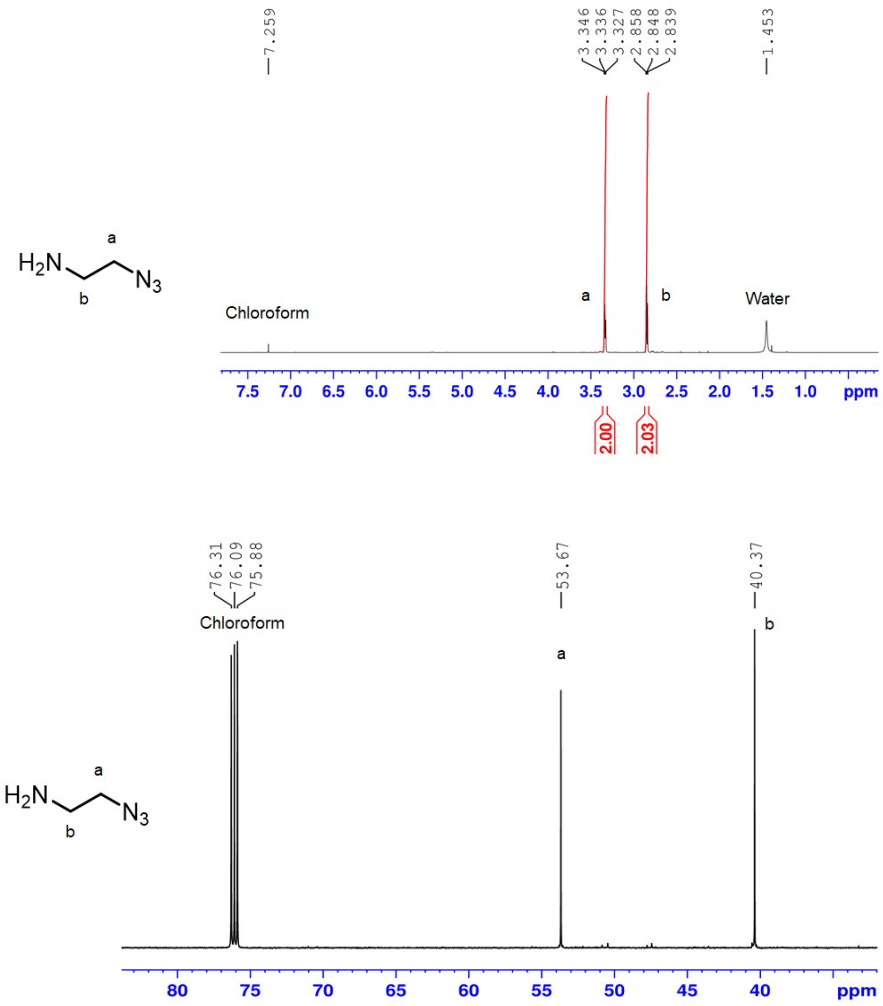


Synthesis of 3,4-dibromo-1-(prop-2-yn-1-yl)-2,5-dihydro-1H-pyrrole-2,5-dione: DB-Alk was synthesized using a published procedure.[1] Propargylamine (0.025 mL, 0.381 mmol) was added to a solution of compound 2 (0.100 g, 0.432 mmol) in 5 mL of DCM and stirred for 30 min. After stirring, 40 mL of DCM was added, and the organic phase was dried over anhydrous $MgSO_4$, filtered, and solvent was removed under reduced pressure. Yield (0.097g, 0.331 mmol, 0.387%) ^{1}H NMR (600 MHz, $CDCl_3$) δ ppm 2.27 (1H, s, J=2.43 Hz), 4.38 (2H, d, J=2.46 Hz). ^{13}C NMR (600 MHz, $CDCl_3$) δ ppm 28.61, 72.58, 76.06, 129.83, 162.66. HRMS: Mass calculated for $[M+H]^+$. 291.8609, observed 291.8604.



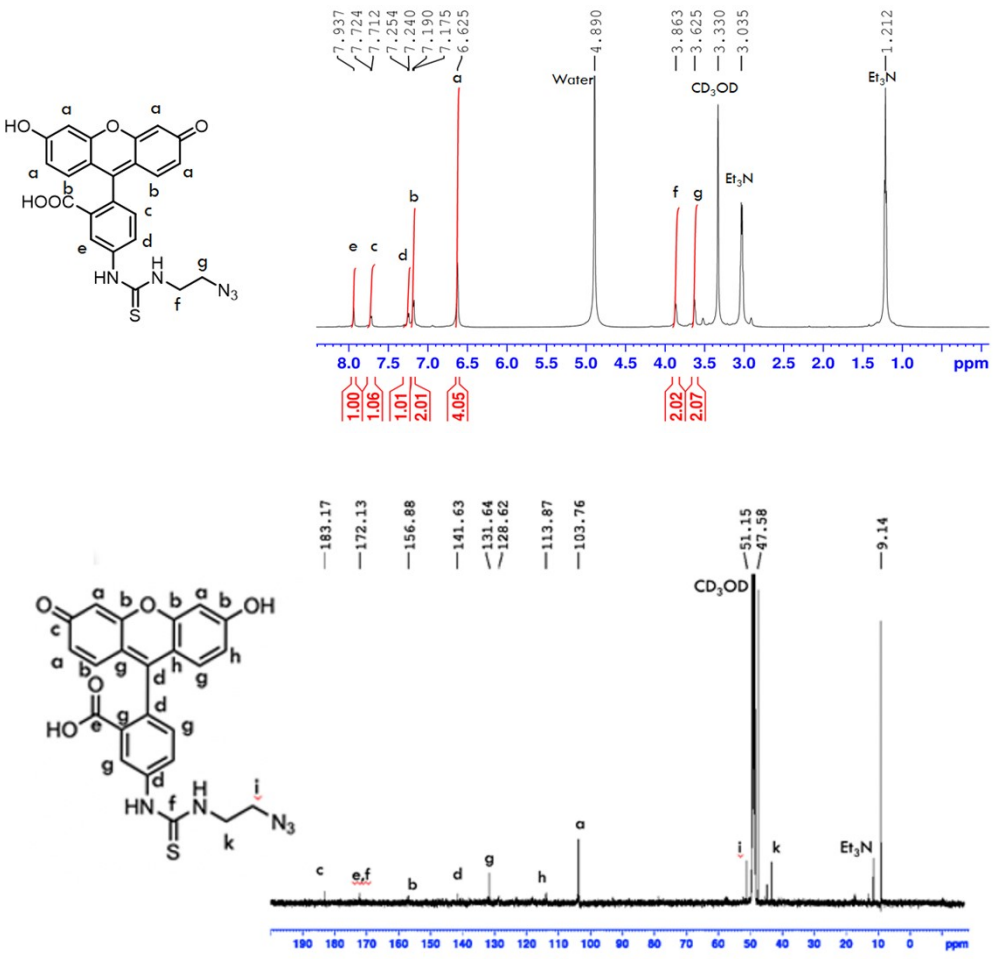
^{1}H and ^{13}C NMR of DB-Alk.

Synthesis of 2-azido-ethylamine*:* A sodium azide solution (3.1 g, 47 mmol) in 13 ml of water was mixed with 2-Bromoethylamine hydrobromide (3.1 g, 15 mmol) and heated up to 80 °C for 24 h. Then the mixture was cooled down to 0 °C in an ice bath and kept for 15 minutes to make the temperature homogenous in all parts of the mixture. Then, KOH (4.0 g, 71 mmol) was added to the stirred solution at 0° C. The mixture was then extracted with diethyl ether (4 ×16 mL). The organic phase was collected and dried over $MgSO_4$ and filtered. The solvent was removed under reduced pressure at 35 °C to yield 1.18 g (13.7 mmol, 71%) of 2-azido-ethylamine. ^{1}H-NMR ($CDCl_3$, 600 MHz) δ: 3.35 - 3.55 (t, 2 H), 2.84 – 2.86 (t, 2 H). ^{13}C-NMR ($CDCl_3$, 600 MHz) δ: 40.37, 53.67.



^{1}H and ^{13}C NMR 2-azido-ethylamine.

Synthesis of fluorescein-azide: 5-(3-(2-Azidoethyl)thioureido)-2-(6-hydroxy-3-oxo-3H-xanthen-9-yl)-benzoic acid [FITC-Azide] was synthesized as reported in the literature.[3] A 15 mL MeOH solution of fluorescein isothiocyanate (150 mg, 0.77 mmol) was mixed with 500 µL of TEA and 2-azidoethyl amine (65.0 mg, 0.535 mmol). The mixture was then stirred overnight at room temperature. The solvent was evaporated under vacuum and the dark orange powder was collected (0.115 g, 0.242 mmol, 81% yield).). ^{1}H-NMR (CD_3OD, 600 MHz) δ: 7.93 (s, 1 H), 7.71 – 7.72 (d, 1 H), 7.24 – 7.25 (d, 1 H), 7.17 – 7.19 (d, 2 H), 6.62 (s, 4 H), 3.86 (s, 2 H), 3.62 (s, 2 H).^{13}C-NMR (CD_3OD, 500MHz) δ: 9.14, 47.58, 51.15, 103.76, 113.87, 128.62, 131.64 , 141.63, 156.88, 172.13, 183.17.



^{1}H and ^{13}C NMR of *fluorescein-azide.*

Determination of conjugation yield using Ellman's assay: Ellman's reagent solution was prepared by dissolving 4 mg of Ellman's reagent in 1 mL of reagent buffer (0.1 M sodium phosphate buffer, pH 8.00 with 1 mM EDTA). To make a working solution, 125 µL of prepared Ellman's reagent was added to 6.25 mL of reagent buffer. 25 µL of cysteine standards (0 mM, 0.25 mM, 0.5 mM, 0.75 mM, 1.0 mM, 1.25 mM, 1.5 mM) was added into 255 µL of Ellman's working solution, followed by incubation at RT for 15 min before reading the absorbance at 500 nm. A standard curve was plotted (Figure S11). The unfunctionalized cysteine concentrations of Qβ conjugates were obtained from the standard curve. The reaction yield was calculated using:

$$Yield\% = \left(1 - \frac{c_1}{c_2}\right) * 100\%$$

c_1: cysteine concentration of Qβ conjugates

c_2: cysteine concentration of Qβ before reaction

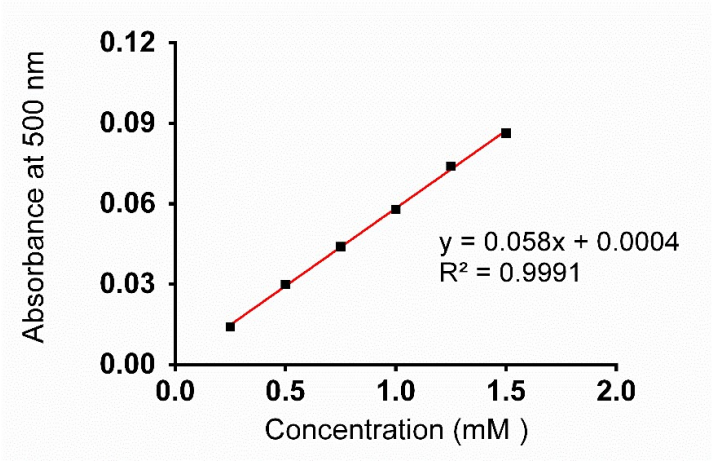


Figure S1. Ellman's assay calibration curve. The standards cysteine concentrations are 1.5 mM, 1.25 mM, 1.0 mM, 0.75 mM, 0.5 mM, 0.25 mM, and 0 mM in Ellman's assay reagent buffer (0.1 M sodium phosphate buffer, pH 8.0 with 1 mM EDTA)

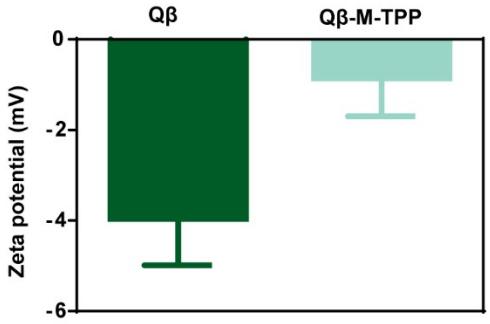


Figure S2. ζ potential variation of Qβ before and after M-TPP conjugation

Labeling Qβ-Maleimide with FITC (Qβ-M-FITC): To label Qβ-Maleimide with FITC to test cytoplasm mimicking experiment by monitoring FITC fluorescence, first Qβ is decorated with dibromomaleimide-alkyne (DB-Alk). 3 mg stock of purified Qβ was reduced using 10 eq. of TCEP HCl (1h, RT). To the solution of reduced Qβ, 2 mL of 0.1 M NaP buffer (pH 5) and 20 eq. of DB-Alk in DMF (20 µL) was added, which cause the appearance of a bright yellow fluorescence under a TLC lamp. The solution was allowed to sit at RT, overnight. The resulting solution was washed (3×) in a 10 K MWCO centrifugal spin column with 0.1 M NaP buffer (pH 5) three times. Next, FITC-azide was conjugated to the alkyne group on the Qβ surface through copper-catalyzed azide-alkyne cycloaddition (CuAAC) click chemistry. To do that, 10 µL of FITC-N3 in DMF (16.2 mM) was added to 1 ml of 1 mg/ml Qβ-M-alkyne then 15 µL of CuSO4·5H2O (5 mg, 4 mM) was premixed with 30 µL THPTA (22.0 mg, 41.5 mM) and added to the reaction mixture. Lastly, 150 µL of aminoguanidine HCl (11.0 mg, 99.5 mM) was added, followed by 150 µL of sodium ascorbate (2.00 mg, 13.3 mM). The solution was mixed for 4 h on a rotisserie. The resulting solution was washed (3×) in a 10 K MWCO centrifugal spin column with 0.1 M potassium phosphate buffer (pH 7).

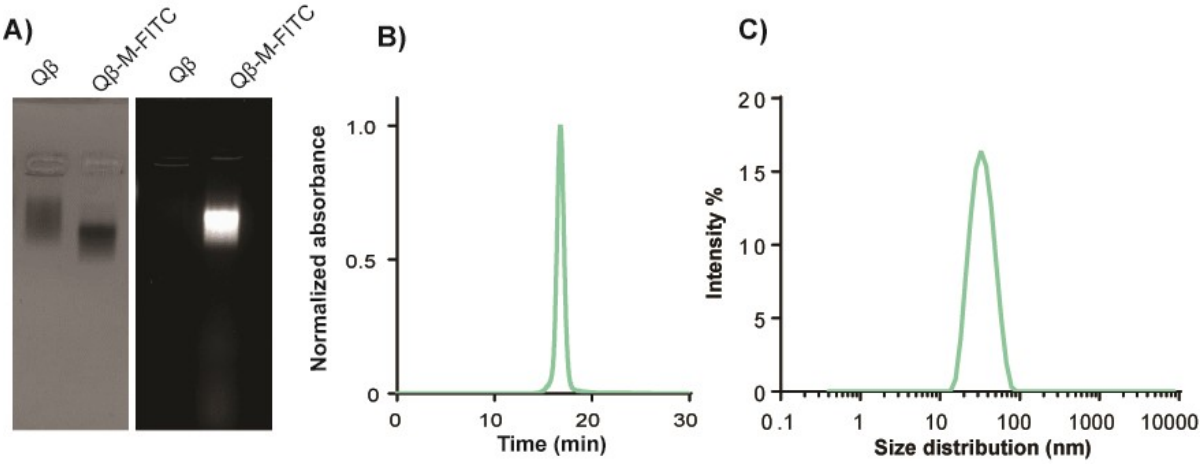


Figure S3. Characterization of Qβ-M-FITC conjugate using A) 1% agarose gel electrophoresis, showing a shift toward positive electrode due to the negative charge of FITC B) SEC and C) DLS shows the unchanged size distribution of 31.70 nm ± 11.7 with PDI: 0.103.

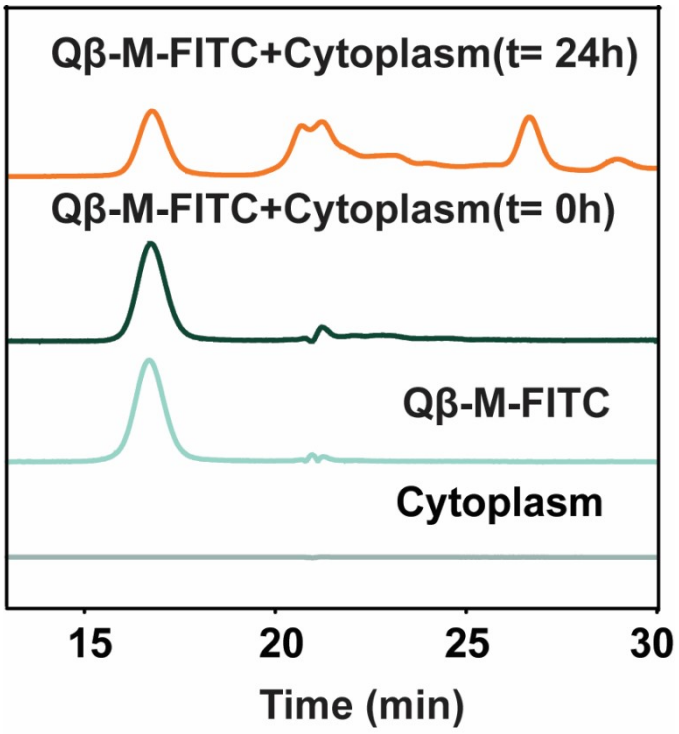


Figure S4. Mimicking the cytoplasm cleavage by subjecting Qβ-M-FITC to a solution of 20 mM HEPES, 100 mM KCl, 1 mM $MgCl_2$, 1 mM EDTA, pH 7.4, and 1 mM glutathione. The mixture was vortexed for 10 s and then maintained at 37 °C for 24 h. M-FITC release was monitored by size exclusion chromatography.

Artificial cytoplasm cleavage: After a successful conjugation of the maleimide-FITC linker to Qβ, the cytosolic cleavage ability of the Qβ-M-FITC system was checked *ex vitro*. A solution that would approximate the cytoplasm (20 mM HEPES,100 mM KCl, 1 mM $MgCl_2$, 1 mM EDTA, pH 7.4, 1 mM, glutathione, 37 °C) was prepared. Cytoplasm solution (20 µl) was added to a solution of Qβ-M-FITC (380 µl of 0.2 mg/ml) and incubated at 37 °C for 24 h. Cleavage of M-FITC from Qβ was monitored by size exclusion chromatography at 490 nm.

Fluorescence microscopy studies*:* 1×10^5 A549 cells were seeded on a 35 mm glass bottom petri dish (MatTek) in DMEM (Sigma-Aldrich, supplemented with 10% FBS, 4500 mg/L glucose, and 1% Pen-Strep) cell culture media, and incubated at 37 °C, 5% CO_2 one day before experiments. 2 mg/ml of Qβ(GFP) and conjugate formulations were prepared in serum-free cell media and incubated with cells at 37 °C, 5% CO_2 for 4 hours. Cells were washed three times with serum-free cell media and three times with 1× DPBS, followed by nucleus staining (DAPIThermo Fisher Scientific) or LysoTrackerat 37 °C for 30 min. Cells were then washed with 1×DPBS three times before imaging. All the samples were imaged using an epifluorescence EVOS fl microscope. Filter setting: GFP-UV (laser: 405 nm, filter: 500 nm to 540 nm) (Qβ (GFP) and Qβ GFP) conjugates), Cy5 (nucleus). Images were processed using ImageJ.

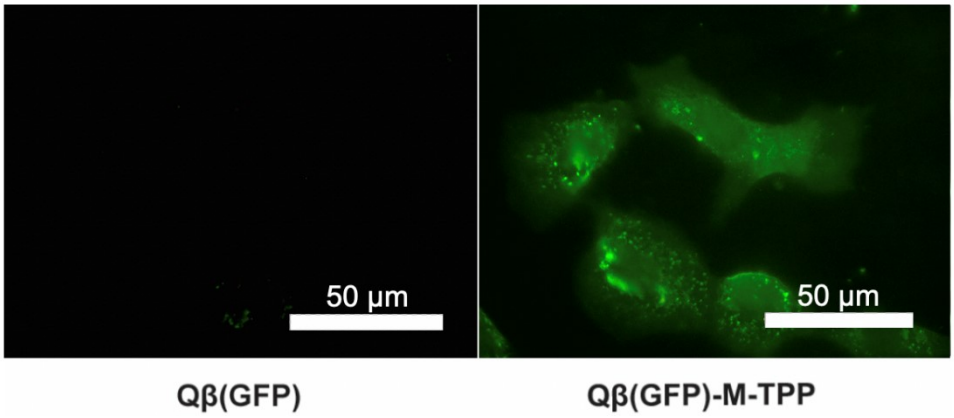


Figure S5. Fluorescence micrographs of Qβ(GFP) and Qβ(GFP)-M-TPP in live A549 cells where the Qβ(GFP)-M-TPP shows entry into the cytosol, while the green fluorescence in Qβ(GFP) is quenched by the acidic environment of endosome. Color code: green: Qβ(GFP). Scale bar = 50 µm.

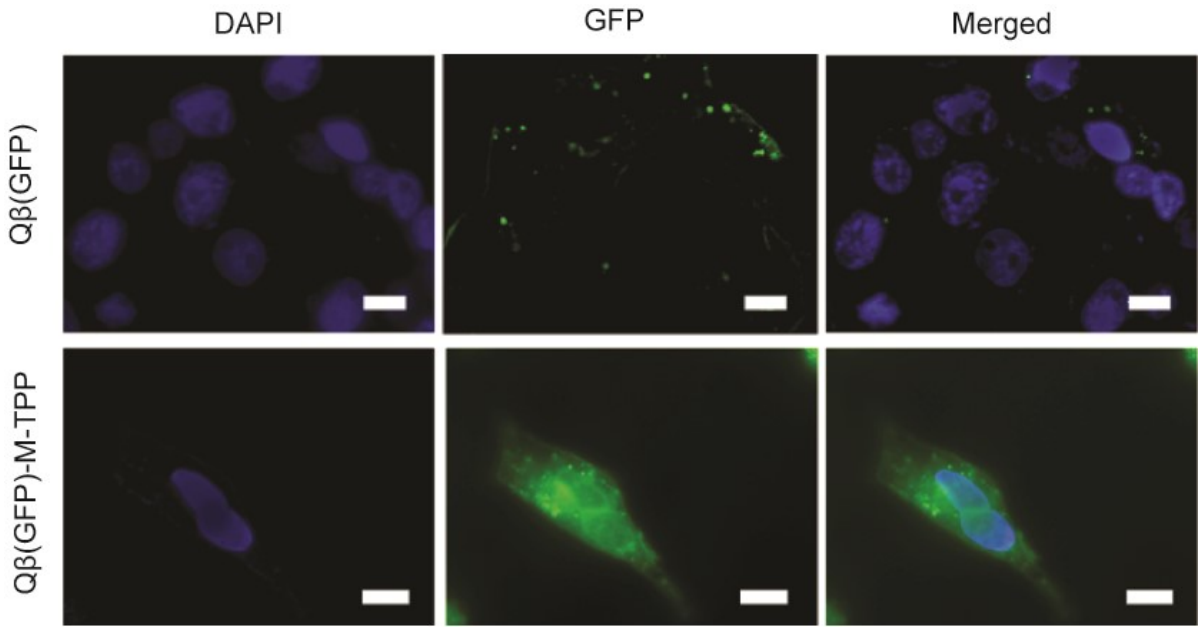


Figure S6. Nuclei staining of A549 cells treated with Qβ(GFP)-DB-TPP and Qβ(GFP). Fluorescence micrograph showing cytosolic delivery of Qβ(GFP)-DB-TPP. Scale bar:10µm.

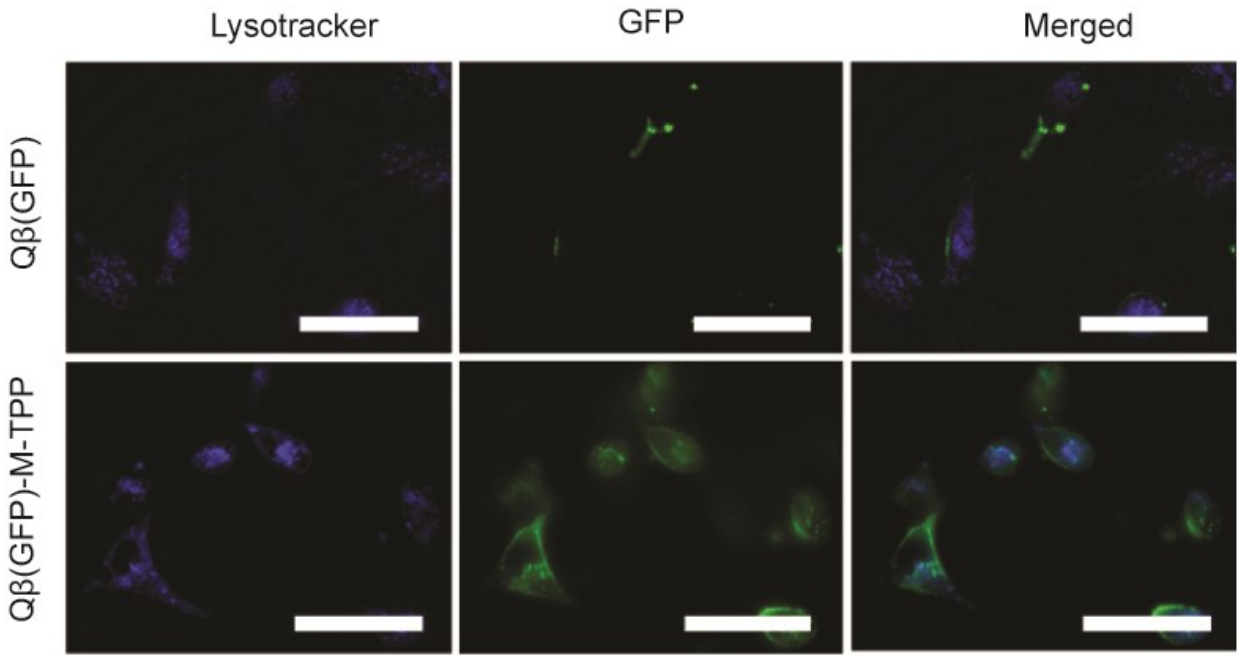


Figure S7. Fluorescence micrograph of A549 cells with Qβ(GFP)-TPP and Qβ(GFP). Lysotracker in blue; particles in green. Scale bar = 50 μm.

Ex vivo fluorescence microscopy study: The focus of this experiment is to show that the fluorescent smURFP@Qβ-M-TPP can better escape endolysosome degradation compared to Qβ. To determine this, 300 μL of 2000 μg mL^{-1} of smURFP@Qβ-M-TPP and smURFP@Qβ were prepared in PBS buffer, and were injected through lavage to the lungs of BALB/c mice (N=3) 3x 100 μL 30 min apart for a total injection of 300 μL. At 6 h post-injection, mice were sacrificed (isoflurane asphyxiation, followed by cervical dislocation), and the lungs were collected. The lungs were washed with PBS and fixed in 4% formaldehyde for 48 h. The tissue was prepped for paraffin embedding through first washing with PBS (3x) and then in 50% EtOH: H_2O solution for 24 h, then processed in a fluid transfer processor (dehydration method followed by alcohol washes). Then, using a Tissue-Tek TEC, the tissue was embedded in paraffin and left to sit for 24 h. The samples were sliced at 5 μm using a Leica rotary microtome and collected on slides. After sitting for 24 h at RT, they were prepped through washes with xylenes (x2) and then a gradient of 100% EtOH to 30% EtOH in H_2O, and washed with PBS. FluoroQuest Anti-Fading Mounting Medium with DAPI (20 μL per slide) was applied and the tissue was analyzed through a confocal Zeiss AXIO Imager.Z2 Motorized Fluorescence Microscope.

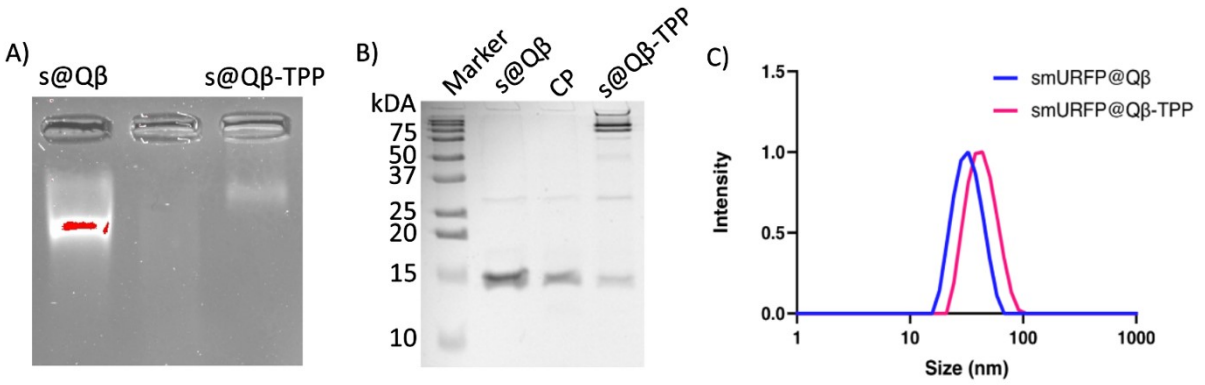


Figure S8. A) Agarose (1%) analysis of pre- and post-TPP modification of genetically modified smURFP@Qβ (s@Qβ) with fluorescence being observed from the smURFP. The upward migration of the smURFP@Qβ-TPP band is due to the positive charge of the TPP. B) Coomassie-stained 18% SDS-PAGE of smURFP@Qβ (processed t, coat protein (CP), and re-bridging of smURFP@Qβ with TPP — as observed by the hexamer band in the sample. C) DLS shows the minimally changed size distribution of the Qβ from ~33 nm (PDI: 0.053) to ~43 nm (PDI: 0.100).

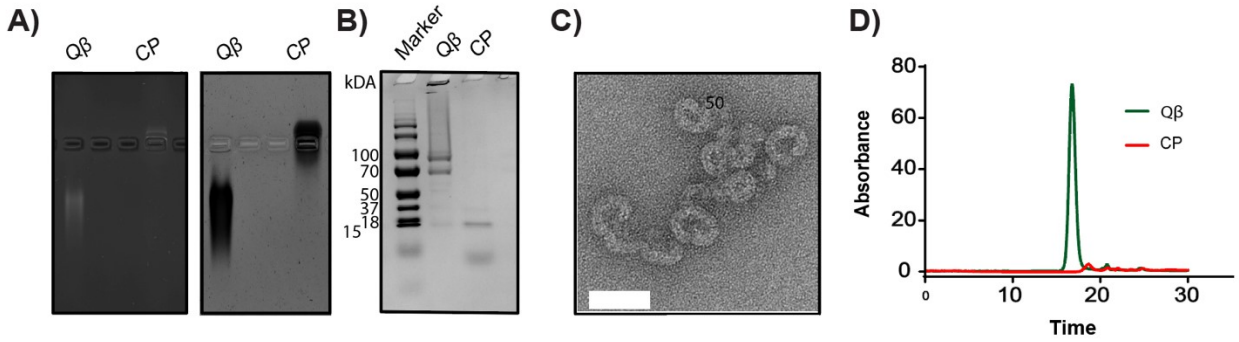


Figure S9. Characterization of disassembled Qβ. A) Native agarose gel electrophoresis SYBRGold stained showing removing *E. coli* RNA in Qβ, (Right) Protein-stained bands show a positive nature for CP by moving toward negative electrode vs Qβ B) Coomassie-stained 10% SDS-PAGE of Qβ and coat protein showing reduction of crosslinked hexameric and pentameric subunit after disassembly. C) TEM micrographs of disassembled Qβ display denatured capsid (scale bar: 100 µm). D) Size-exclusion chromatography profile of Qβ and CP analyzed by absorbance at 280 nm.

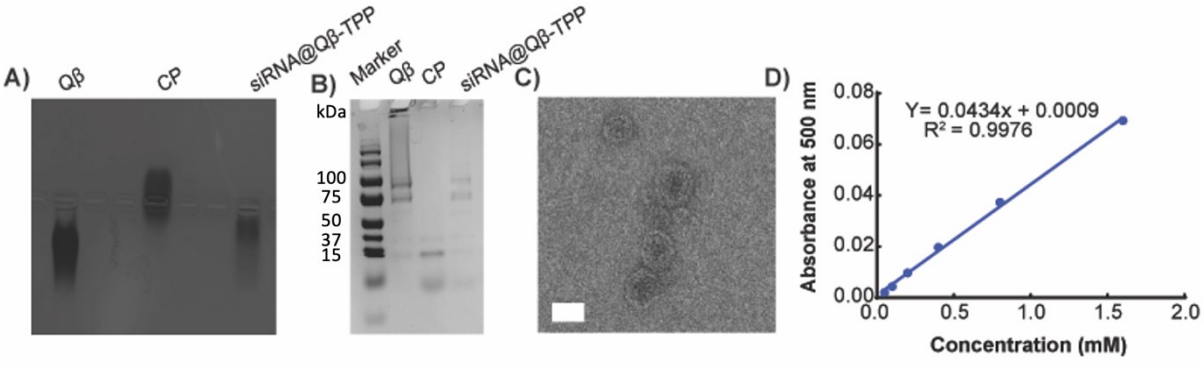


Figure S10. A) protein-stained native agarose (1%) analysis of reassembled, disassembled, and intact Qβ showing clear reformation of VLP band after reassembly. B) Coomassie-stained 10% SDS-PAGE of Qβ, CP and siRNA@Qβ-TPP. Through the protein band integrity and moving toward positive electrode C) Transmission electron microscopy imaging revealed that reconstituted Qβ-M-TPP carrying siRNAs structurally formed unchanged icosahedral particles. (scale bar: 20 μm) D) Cysteine standard curve related to Ellman assay 78% conjugation efficiency is calculated.

1. C. Benjamin, Z. Chen, O. Brohlin, H. Lee, A. Shahrivarkevishahi, S. Boyd, D. Winkler, J.J. Gassensmith, *Nanoscale*, **2020**,12, 9124-9132
2. C Gomes, A., Roesti, E. S., El-Turabi, A., & Bachmann, M. F., Type of RNA Packed in VLPs Impacts IgG Class Switching-Implications for an Influenza Vaccine Design. *Vaccines* **2019,** *7* (2), 47.
3. Zhuo Chen, S. D. B., Jenifer S. Calvo, Kyle W. Murray, Galo L. Mejia, Candace E. Benjamin, Raymond P. Welch, Duane D. Winkler, Gabriele Meloni, Sheena D'Arcy, and Jeremiah J. Gassensmith, Fluorescent Functionalization across Quaternary Structure in a Virus-like Particle. *Bioconjugate Chem* **2017,** *28* (9), 2277–2283.