

Supporting Information

Mitochondria-specific Conjugated Polymers Nanoparticles

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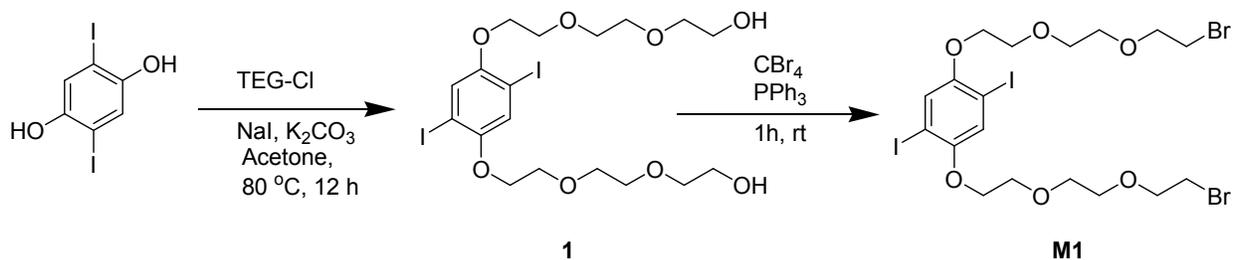
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1.0 General Information

Chemicals, including solvents, were purchased from Fisher Scientific and used as received. Deuterated solvents were purchased from Cambridge Isotope Laboratories (Cambridge, MA). UV-vis spectra were recorded using a Varian Cary 50 Bio spectrophotometer. Fluorescence spectra were obtained using a FluoroLog-3 Spectrofluorometer (Jobin Yvon/Horiba). 9, 10-diphenylanthracene (QY = 1.0) in cyclohexane was used as a fluorescence standard for QY determination. The average molecular weight (M_n) and polydispersity ($PDI = M_w/M_n$) of the polymers were determined by gel permeation chromatography (GPC) against polystyrene standards using a Shimadzu high performance liquid chromatography (HPLC) system fitted with PLgel 5 μ m MIXED-D columns and SPD-20A ultraviolet-visible (UV-vis) detector. Nuclear magnetic resonance (NMR) spectra were recorded on a 400 MHz Avance Bruker NMR spectrometer. ^1H and ^{13}C NMR chemical shifts are given in ppm relative to $\text{Si}(\text{CH}_3)_4$, with the solvent resonance used as an internal reference. ^1H NMR on the scale based on the middle peak ($\delta = 2.50$ ppm) of the dimethyl sulfoxide DMSO-d_6 solvent as an internal standard or 7.26 ppm for chloroform (CDCl_3) experiments. ^{31}P NMR chemical shifts are reported in ppm relative to H_3PO_4 . The mass spectrometric data were obtained at the mass spectrometry facility of Florida International University. Analytical thin layer chromatography (TLC) was performed on TLC Silica gel 60 F254. The TLC plates were visualized by shortwave (254 nm) or longwave (360 nm) UV light. Flash chromatography on silica gel (230–400 mesh) was performed. Fourier transform infrared (FT-IR) spectrum was obtained on a PerkinElmer Spectrum 100 FT-IR Spectrometer; fine powder sample was mounted on an attenuated total reflection cell.

2.0 Monomer Synthesis

Scheme S1:



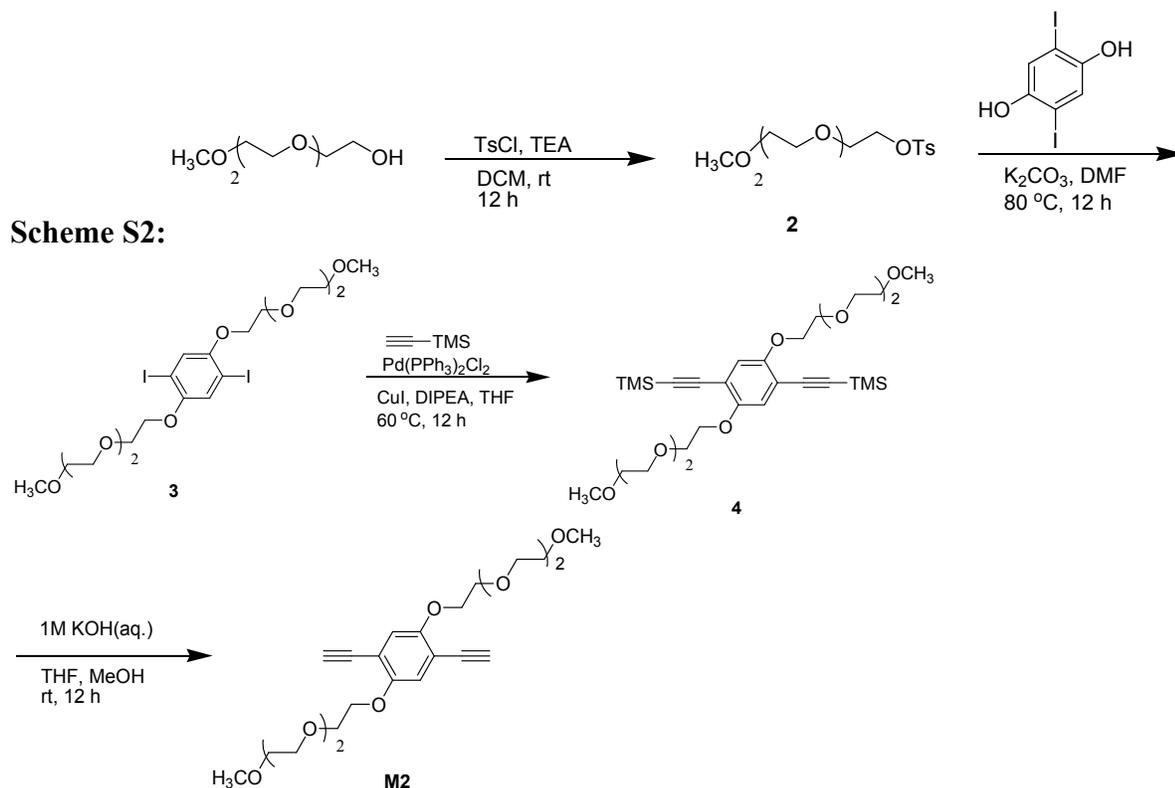
2.1 Synthesis of compound 1¹

1, 4-Dihydroxy-2, 5-diiodobenzene (5.00 g, 13.80 mmol) was dissolved in acetone (20 mL) and the resulting solution was added slowly to a stirred suspension of K_2CO_3 (7.60 g, 55.25 mmol) and NaI (8.30 g, 55.25 mmol). The reaction mixture was allowed to stir for 10 min, followed by the slow addition of triethylene glycol monochlorohydrin (TEG-Cl) (5.80 g, 34.53 mmol). The reaction was heated to 80 °C for 12 h. The solution was cooled to room temperature, the solvent was removed, and the resulting slurry was re-dissolved in CH_2Cl_2 (100 mL). The solution was

extracted with water (100 mL x3) and the organic fractions were collected and concentrated in vacuo until an oily residue remained. The crude product was purified by silica gel chromatography (95:5 EA/Hexane). The product was off-white solid. Yield: 5.80 g (67.0%). ¹H NMR (400 MHz, CDCl₃): δ 7.24 (s, 2H), 4.09- 4.15 (t, 4H), 3.86-3.92 (t, 4H), 3.77-3.82 (m, 4H), 3.69-3.76 (m, 8H), 3.60-3.66 (m, 4H), 2.29 (br, 2H); ¹³C NMR (100 MHz, CDCl₃): δ 153.51, 123.21, 86.52, 72.51, 71.22, 70.54, 70.28, 69.66, 61.82.

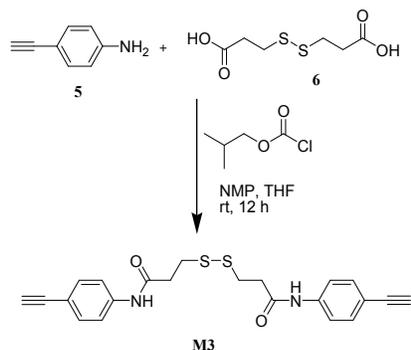
2.2 Synthesis of monomer M1²

A suspension of compound **1** (5.00 g, 7.98 mmol) was dissolved in 25 mL of DCM. Carbon tetrabromide (8.00 g, 23.95 mmol) and triphenyl phosphine (12.60 g, 23.95 mmol) were added slowly to the reaction mass. The mixture was stirred at room temperature for 2 h. The solvent was removed and the crude product was purified by column chromatography (1: 4 EA / Hexane). The product was white solid. Yield: 5.30 g (81.2%). ¹H NMR (400 MHz, CDCl₃): δ 7.30 (s, 2H), 4.10-4.15 (t, 4H), 3.89-3.92 (t, 4H), 3.85-3.87 (t, 4H), 3.68-3.76 (m, 4H), 3.60-3.65 (m, 4H), 3.45-3.51 (m, 4H). ¹³C NMR (100 MHz, CDCl₃): δ 153.37, 123.49, 86.47, 71.30, 71.19, 70.71, 70.33, 69.70, 30.42.



Monomer 2 (**M2**) was synthesized according to literature procedure (Scheme 2)³.

Scheme S3:



2.3 Synthesis of monomer M3

3,3'-Dithiodipropionic acid, **6** (1.00 g, 4.76 mmol) was dissolved in anhydrous THF (20 mL) in a round bottom flask, which was evacuated and filled with nitrogen. *N*-methylmorpholine (0.90 g, 9.52 mmol) was added, and the suspension was allowed to stir until the entire solid was dissolved. Isobutyl chloroformate (1.30 g, 9.52 mmol) was added, and the mixture was allowed to stir at room temperature for 15 min. A solution of 4-ethynylaniline, **5** (1.10 g, 9.52 mmol) in anhydrous THF (10 mL) was prepared under a N₂ atmosphere and transferred into the reaction flask using a cannula. The reaction was allowed to proceed overnight, after which the reaction mixture was filtered. The filtrate was concentrated in vacuo and the resulting solid was precipitated overnight from the DCM / diethyl ether solvent system. Yield: 0.70 g (36.0%). ¹H NMR (400 MHz, DMSO): δ 10.20 (s, 2H), 7.50-7.61 (d, 4H), 7.39-7.41 (d, 4H), 4.07 (s, 2H), 2.99-3.03 (t, 4H), 2.74-2.77 (t, 4H). ¹³C NMR (100 MHz, DMSO): δ 169.53, 139.68, 132.36, 118.94, 116.12, 83.49, 79.65, 36.08, 33.38. HRMS (ESI): Calc for C₂₂H₂₀N₂O₂S₂ [M+Na]⁺: 431.0858; found [M+Na]⁺: 431.0801.

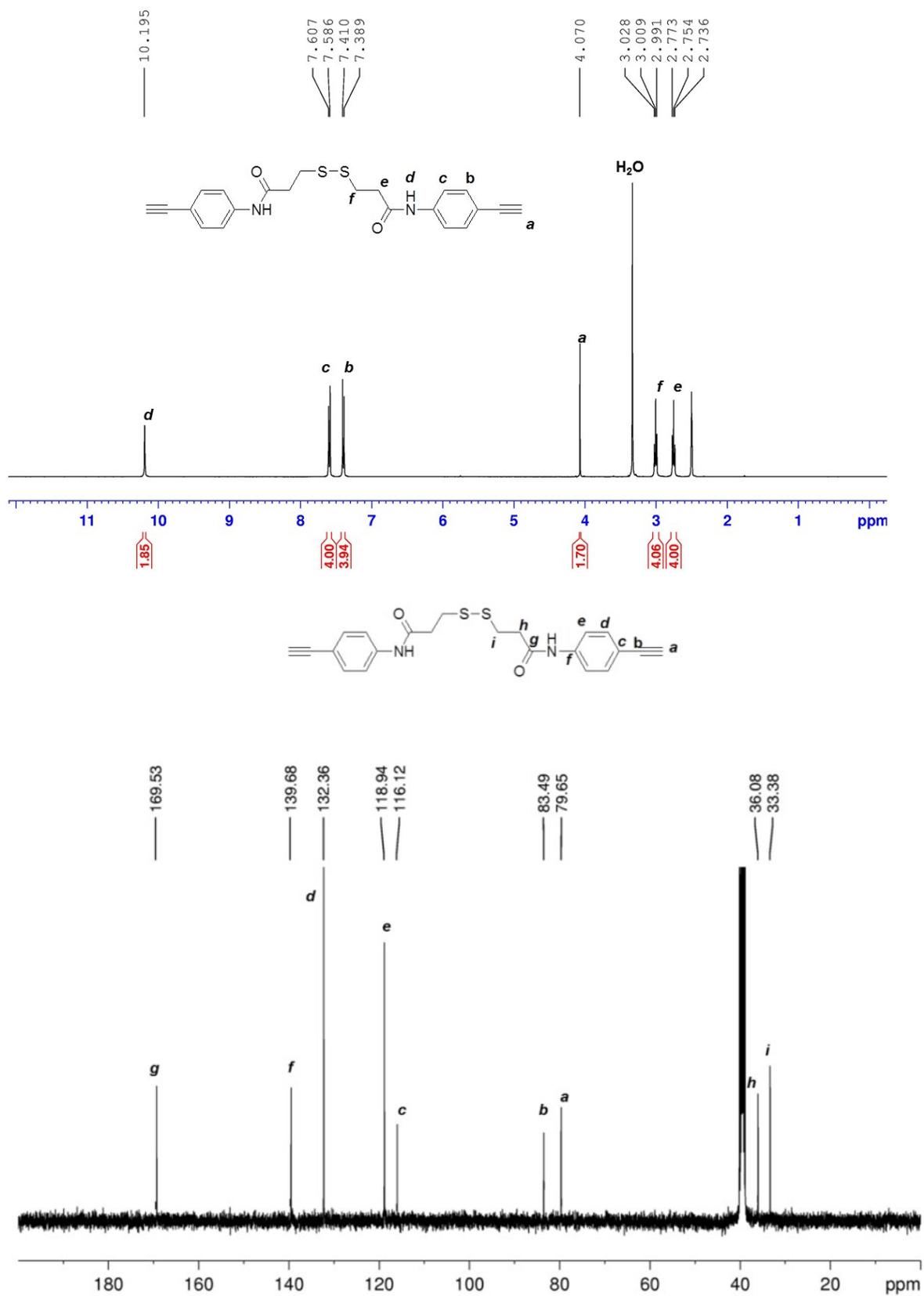


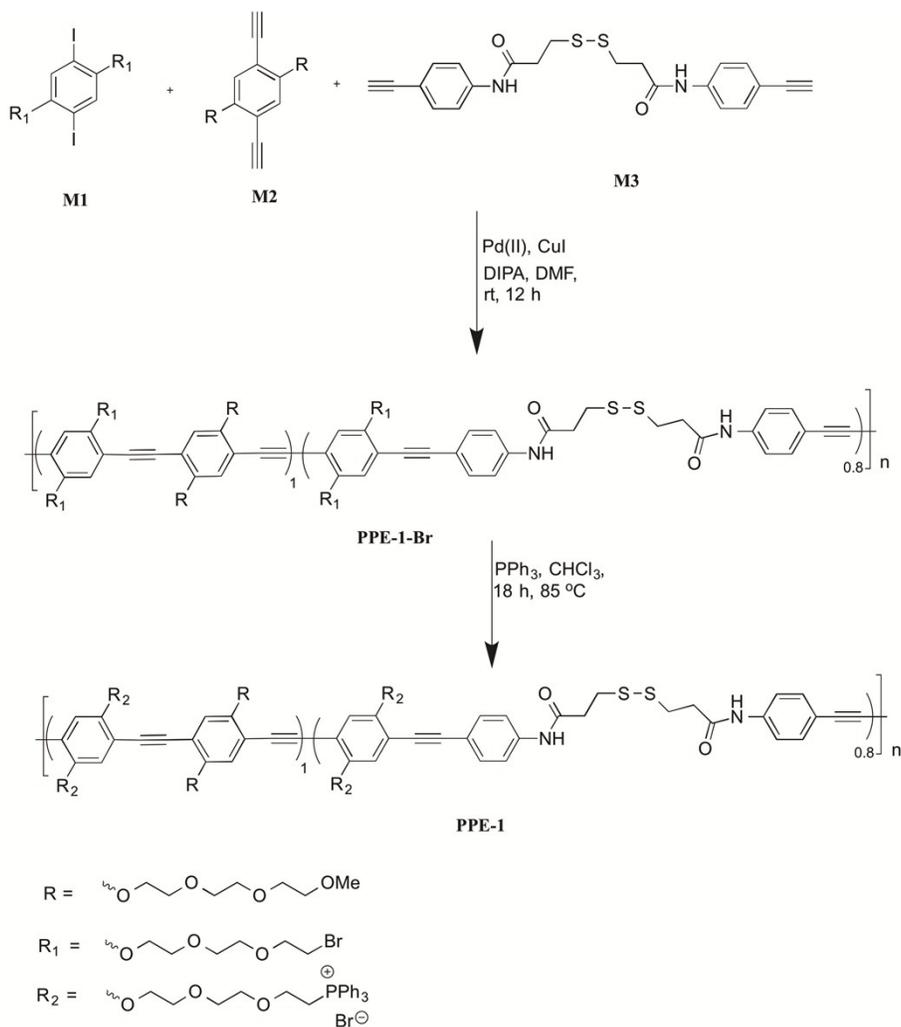
Figure S1. ¹H (top) and ¹³C NMR (bottom) of M3 in DMSO-d₆

3.0 Polymer Synthesis

General Procedure. A Schlenk flask was charged with monomer **M1** (1.00 equiv.), **M2** (0.5 equiv.), **M3** (0.5 equiv.), Pd[(PPh₃)₂Cl₂] (0.10 equiv.), and CuI (0.05 equiv.). The Schlenk flask was evacuated and filled with N₂ and solution of anhydrous dimethylformamide (4 mL) and diisopropylamine (1 mL) was degassed, and 2.5 mL of the mixed solution was transferred to the Schlenk flask using a cannular needle. The reaction was allowed to stir at room temperature for 12 h. The solution was then added drop wise to methanol, resulting in precipitation. After centrifugation (3 min, 4000 rpm) the supernatant was decanted, and the precipitate was redissolved in DCM (1 mL) for further purification.

3.1 Synthesis of PPE-1

Scheme S4:



PPE-1-Br. Using the general procedure described above, the polymerization of monomer **M1** (100 mg, 0.133 mmol), **M2** (30 mg, 0.066 mmol) and **M3** (28 mg, 0.066 mmol) was carried out in presence of Pd[(PPh₃)₂Cl₂] (9.3 mg, 0.013 mmol), and CuI (1.3 mg, 0.007 mmol). The resulting mixture was purified by precipitation in methanol (x3) Yield: 105 mg (42.5 %). An aliquot was redissolved in THF, and the molecular weight was obtained, and photo physical properties were characterized in DCM.

PPE-1-Br: GPC: M_w = 13.66 kDa; M_n = 09.30 kDa; PDI = 1.42. UV-vis: λ_{max} = 400 nm; fluo λ_{max} = 467 nm; QY = 69% and ϵ = 42716.79 M⁻¹ cm⁻¹ (Per repeating Unit) in DCM. ¹H NMR (400 MHz, CDCl₃): δ 7.91 (br, 1H), 7.51 (br, 2H), 7.39 (br, 2H), 6.96 (br, 4H), 4.16 (br, 8H), 3.84 (br, 8H), 3.70 (br, 15H), 3.57 (br, 10H), 3.44 (br, 4H), 3.54 (br, 4H), 3.27 (br, 4H) 3.02 (br, 2H), 2.75 (br, 2H). FT-IR (neat): ν = 2924, 2868, 1671, 1589, 1512, 1408, 1352, 1265, 1214, 1097, 1041, 937, 838 cm⁻¹.

Synthesis of PPE-1.

Triphenyl phosphine (141 mg, 0.540 mmol) was dissolved in chloroform and the resulting solution was added to a stirred suspension of **PPE-1-Br** (100 mg, 0.054 mmol). The reaction was heated to 85 °C for 12 h. The resulting mixture was purified by precipitation in ethyl acetate (x5). Yield: 70 mg (44.7%).

PPE-1: UV-vis: λ_{max} = 405 nm; fluo λ_{max} = 472 nm; QY = 40% and ϵ = 67606.65 M⁻¹cm⁻¹ (Per repeating Unit) in DMSO. ¹H NMR (400 MHz, DMSO-d₆): δ 10.34 (br, 1H), 7.75 (br, 33H), 7.41 (br, 4H), 7.24 (br, 2H), 7.14 (br, 2H), 4.16 (br, 9H), 3.89 (br, 5H), 3.78 (br, 6H), 3.62 (br, 17H), 3.47 (br, 8H), 3.28 (br, 3H), 3.17 (br, 4H), 2.81 (br, 2H). ³¹P NMR (161.9 MHz, DMSO-d₆): 25.38. FT-IR (neat): ν = 2921, 2870, 1683, 1597, 1520, 1430, 1408, 1210, 1099, 1047 cm⁻¹.

CPN-1: UV-vis: λ_{max} = 407 nm; Emission λ_{max} = 470 nm; QY = 14% were measured using 1% DMSO in water (v/v).

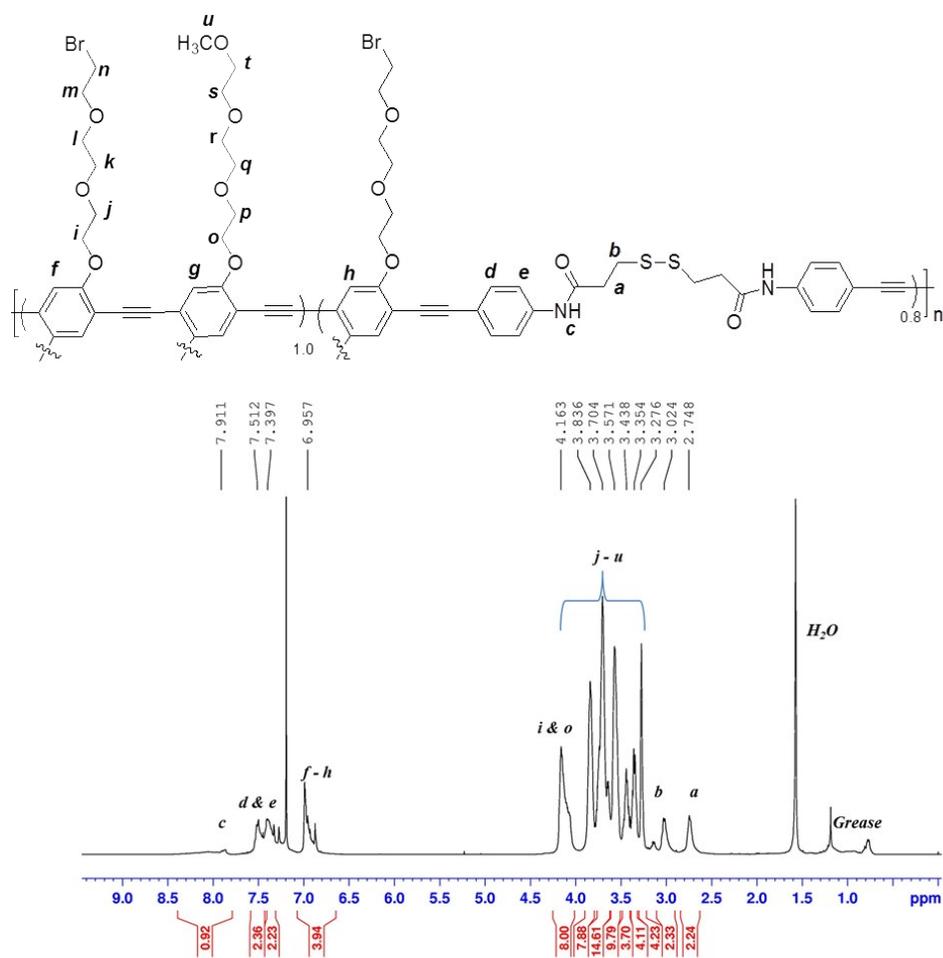
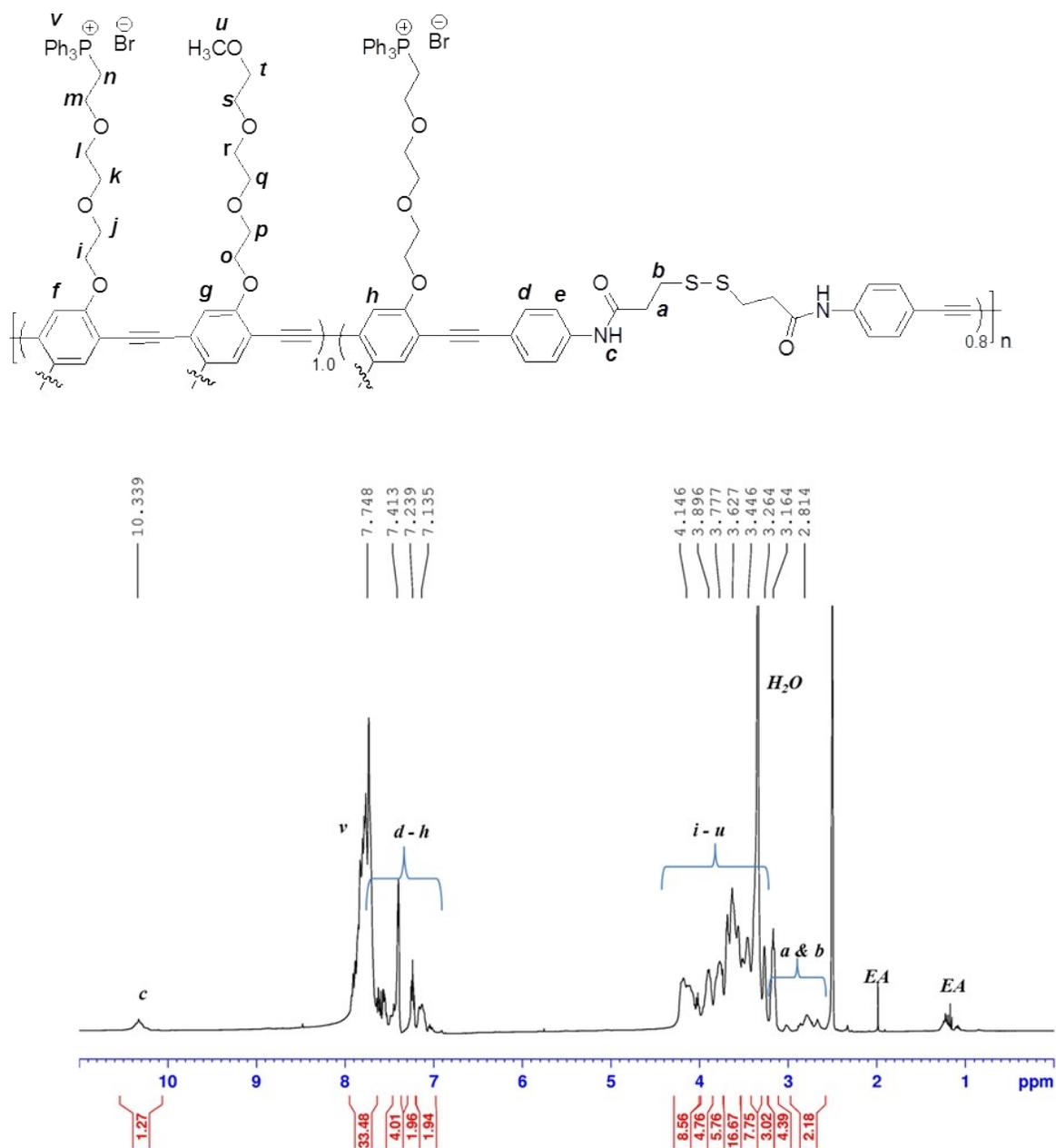


Figure S2. ¹H NMR of PPE-1-Br in CDCl₃.



Fig

re S3. ^1H NMR of PPE-1 in DMSO-d_6 .

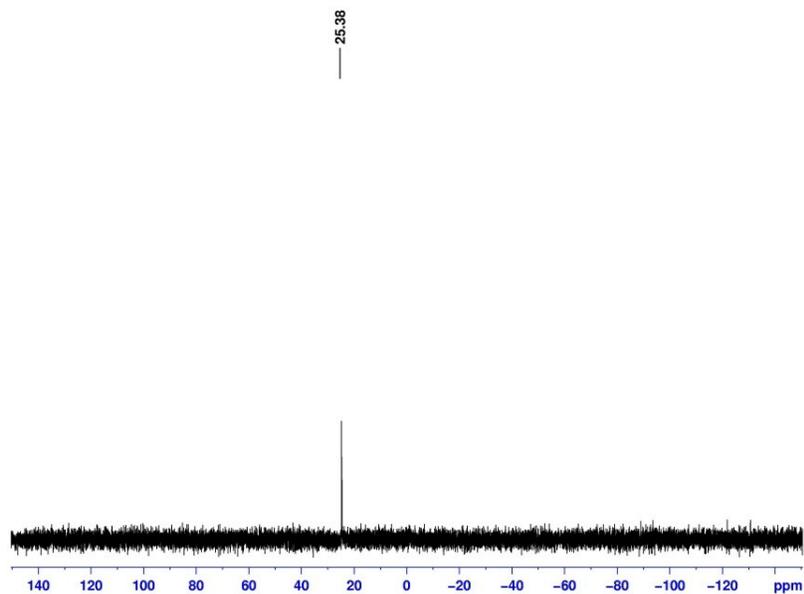


Figure S4. ^{31}P NMR of PPE-1 in in DMSO-d_6 .

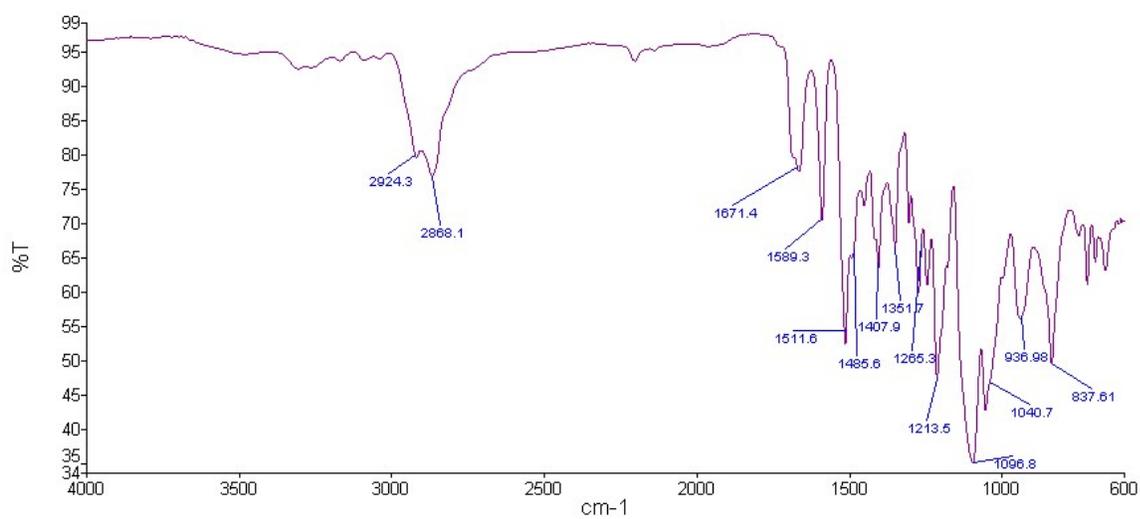


Figure S5. FT-IR of PPE-1-Br (neat).

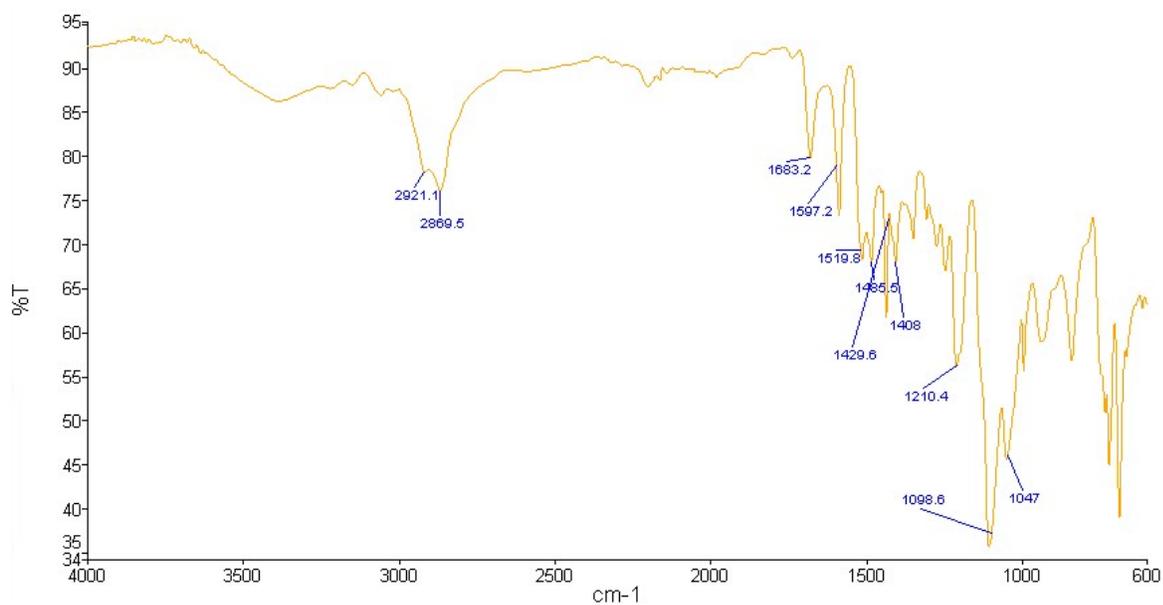


Figure S6. FT-IR of PPE-1 (neat).

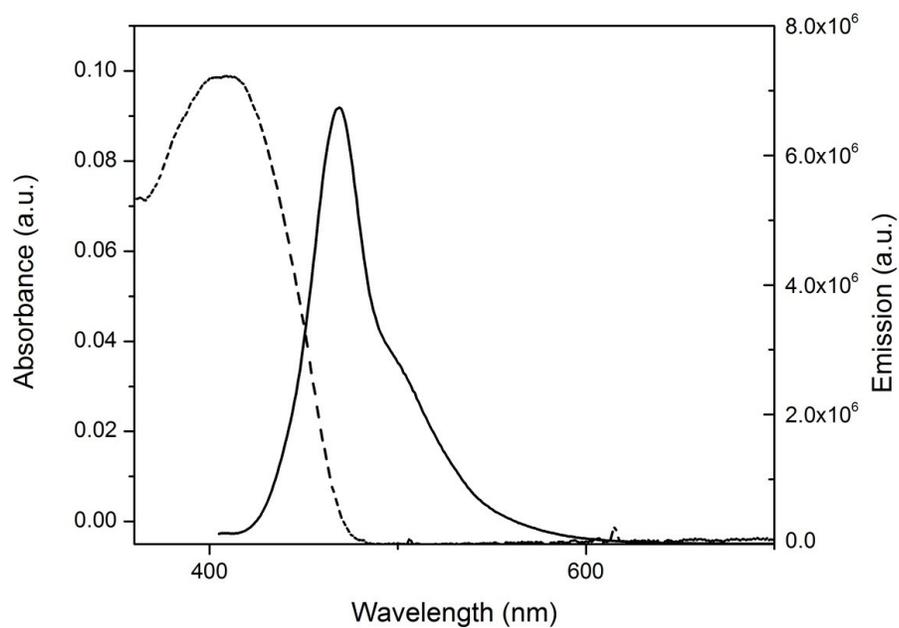


Figure S7. UV-vis and emission spectra of PPE-1-Br in DCM.

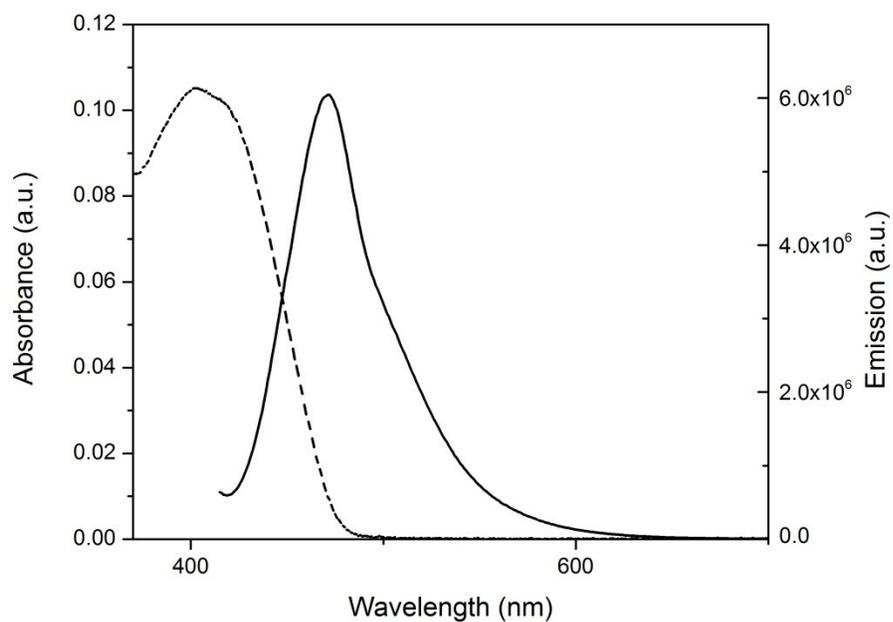


Figure S8. UV-vis and emission spectra of **PPE-1** in DMSO.

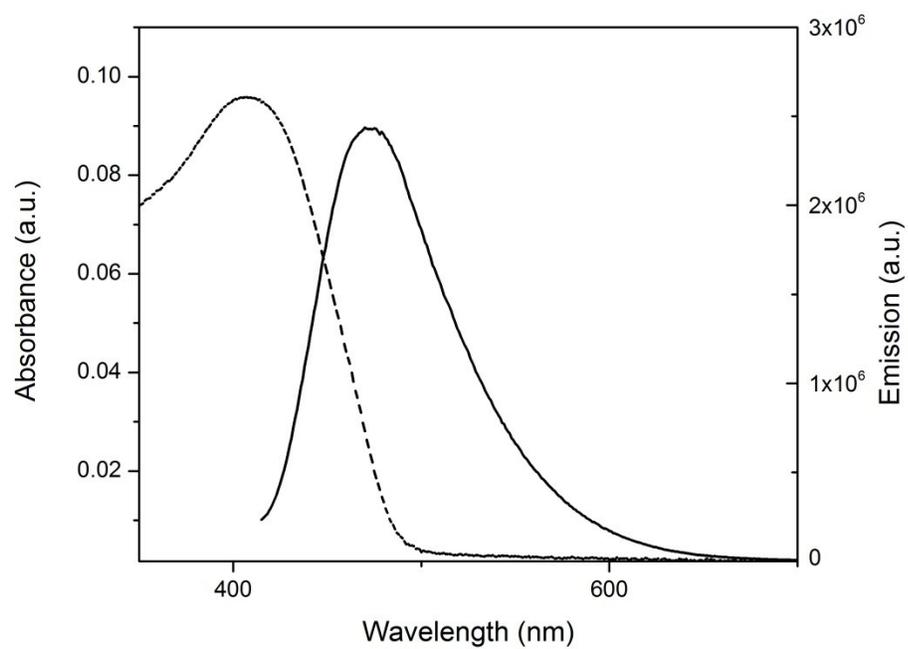
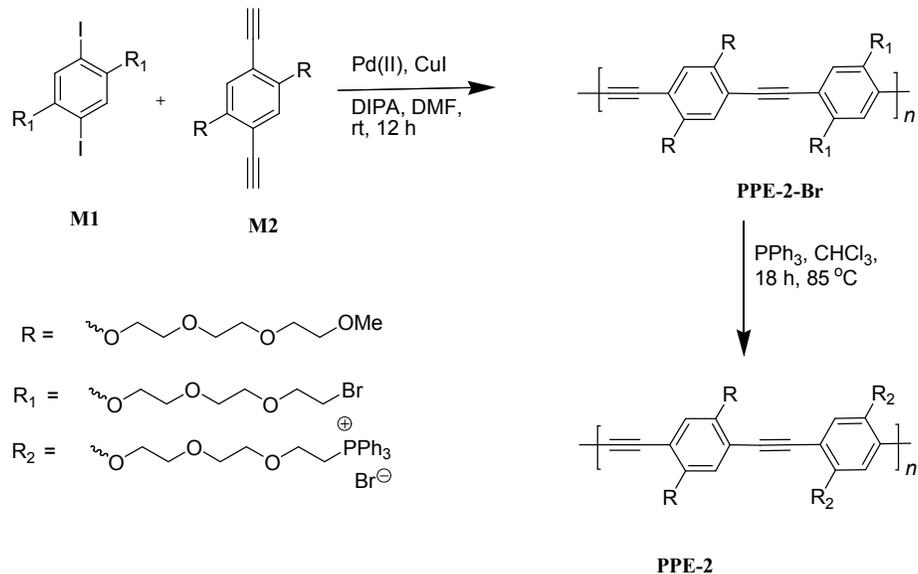


Figure S9. UV-vis and emission spectra of **CPN-1** 1% DMSO in water (v/v).

3.2 Synthesis of PPE-2.

Scheme S5:



PPE-2-Br. Using the general procedure described above, the polymerization of monomer **M1** (100 mg, 0.133 mmol) and **M2** (60.0 mg, 0.133 mmol) was carried out in presence of Pd[(PPh₃)₂Cl₂] (9.3 mg, 0.013 mmol), and CuI (1.3 mg, 0.007 mmol). The resulting mixture was purified by precipitation in methanol (x3) Yield: 0.15 g (88.5 %). An aliquot was redissolved in THF, the molecular weight was obtained, and photo physical properties were characterized in DCM.

PPE-2-Br: GPC: $M_w = 26.36$ kDa; $M_n = 15.98$ kDa; PDI = 1.65. UV-vis: $\lambda_{\text{max}} = 432$ nm; fluo $\lambda_{\text{max}} = 473$ nm; QY = 53% and $\epsilon = 23526.82$ M⁻¹ cm⁻¹(Per repeating Unit) in DCM. ¹H NMR (400 MHz, CDCl₃): δ 7.06 (br, 4H), 4.24 (br, 8H), 3.92 (br, 9H), 3.78 (br, 12H), 3.64 (br, 13H), 3.52 (br, 5H), 3.43 (br, 4H), 3.35 (br, 7H). FT-IR (neat): $\nu = 2923, 2870, 2197, 1510, 1423, 1270, 1241, 1092, 1060$ cm⁻¹.

Synthesis of PPE-2.

Triphenyl phosphine (270 mg, 1.02 mmol) was dissolved in chloroform and the resulting solution was added to a stirred suspension of **PPE-2-Br** (100 mg, 0.102 mmol). The reaction was heated to 85 °C for 12 h. The resulting mixture was purified by precipitation in ethyl acetate (x5). Yield: 95 mg (61.7%).

PPE-2: UV-vis: $\lambda_{\text{max}} = 436$ nm; fluo $\lambda_{\text{max}} = 478$ nm; QY = 25% and $\epsilon = 46128.66$ M⁻¹ cm⁻¹(Per repeating Unit) in DMSO. ¹H NMR (400 MHz, DMSO-d₆): δ 7.75 (br, 30H), 7.15 (br, 4H), 4.13 (br, 8H), 3.91 (br, 4H), 3.77 (br, 6H), 3.61 (br, 16H), 3.45 (br, 12H), 3.27 (br, 4H), 3.15 (br, 10H).

^{31}P NMR (161.9 MHz, DMSO-d6): 24.85. FT-IR (neat): $\nu = 3393, 2874, 2190, 1625, 1493, 1437, 1420, 1353, 1207, 1102, 1046 \text{ cm}^{-1}$.

CPN-2: UV-vis: $\lambda_{\text{max}} = 431 \text{ nm}$; fluo $\lambda_{\text{max}} = 469 \text{ nm}$; QY = 5% were measured using 1% DMSO in water (v/v).

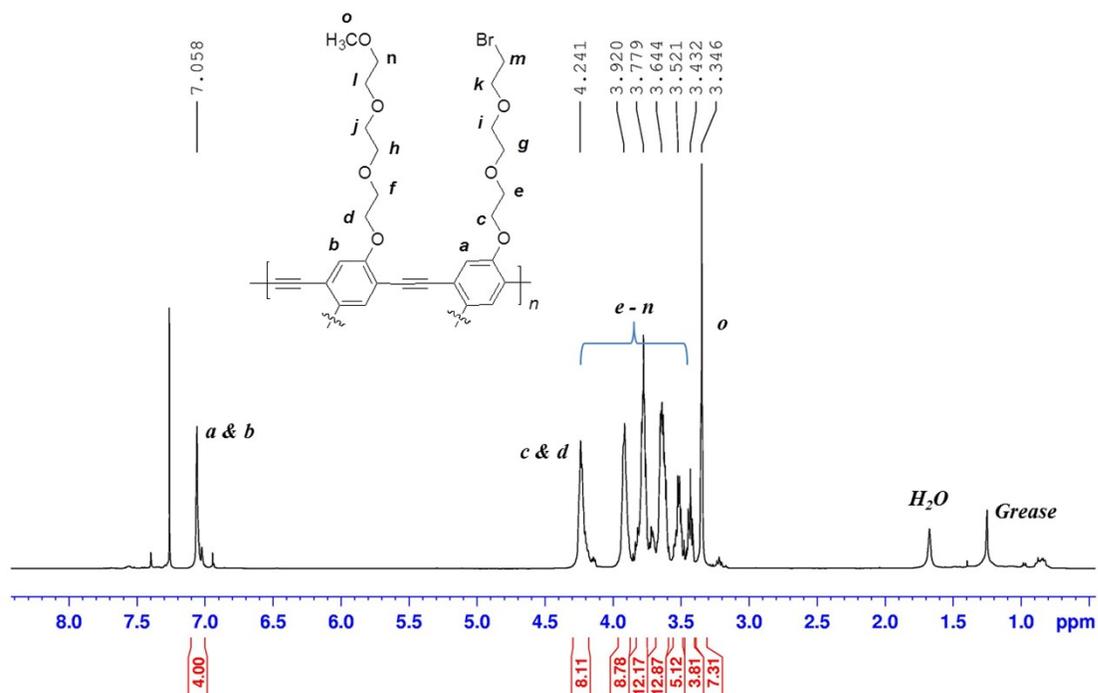


Figure S10. ^1H NMR of **PPE-2-Br** in CDCl_3 .

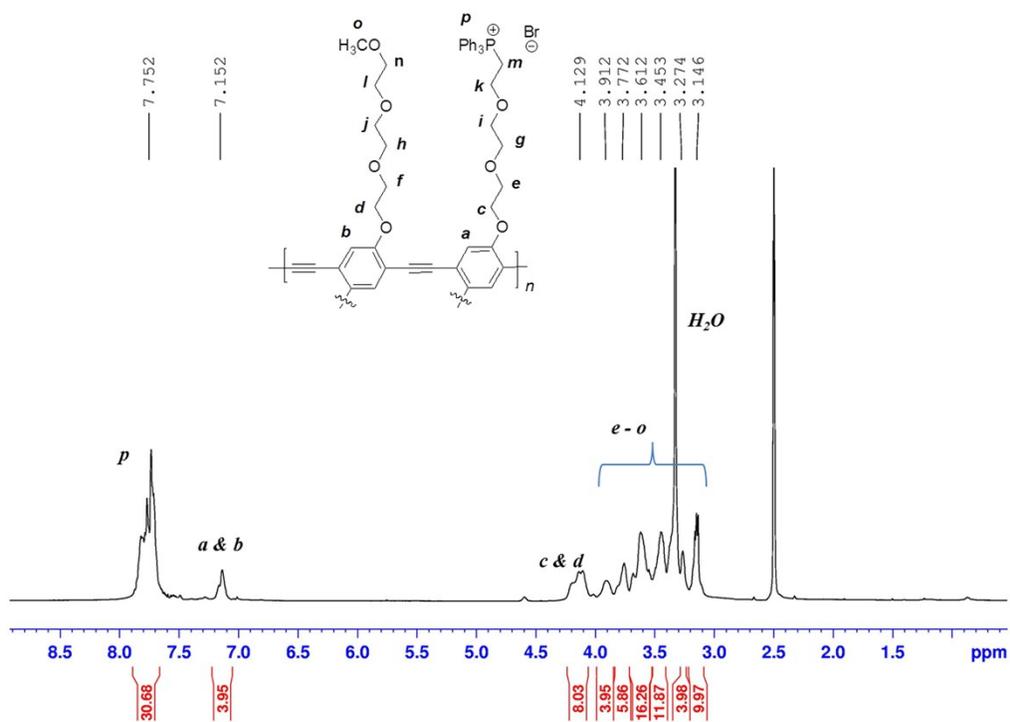


Figure S11. ^1H NMR of PPE-2 in DMSO-d_6 .

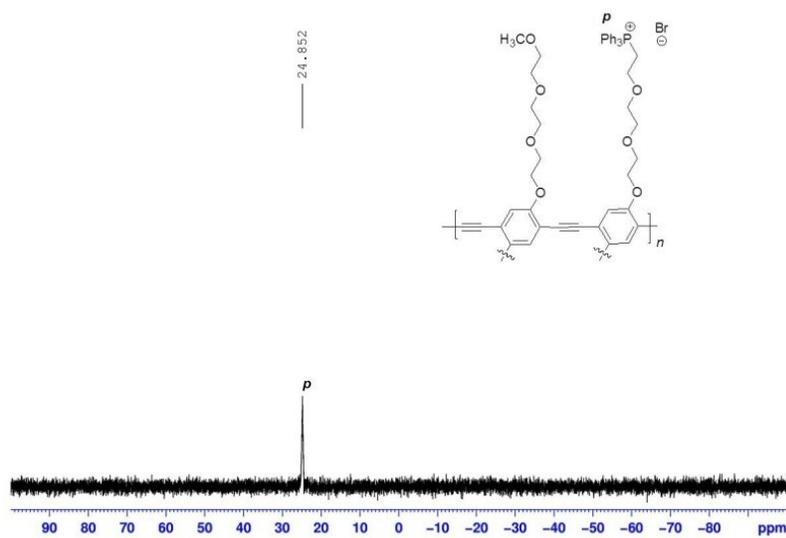


Figure S12. ^{31}P NMR of PPE-2 in DMSO-d_6 .

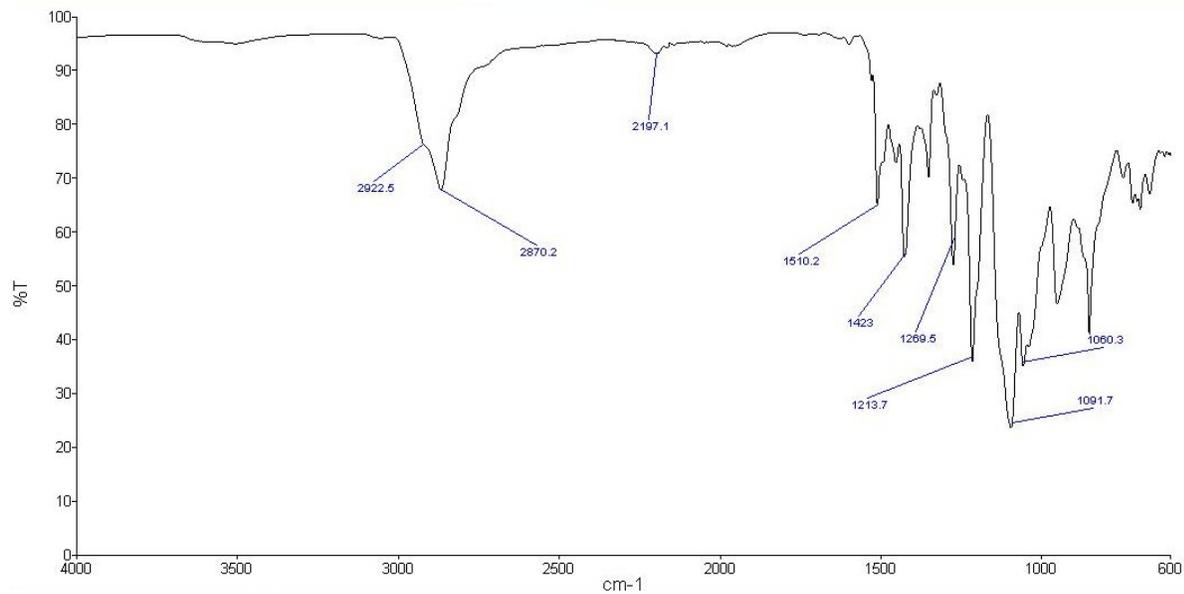


Figure S13. FT-IR of PPE-2-Br (neat).

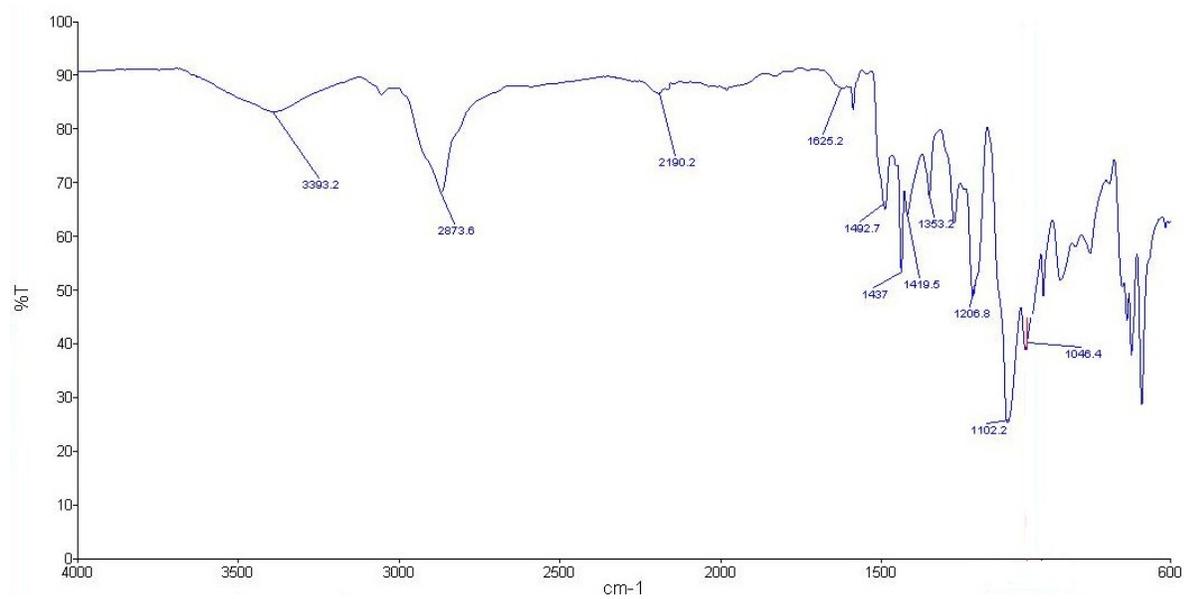


Figure S14. FT-IR of PPE-2 (neat).

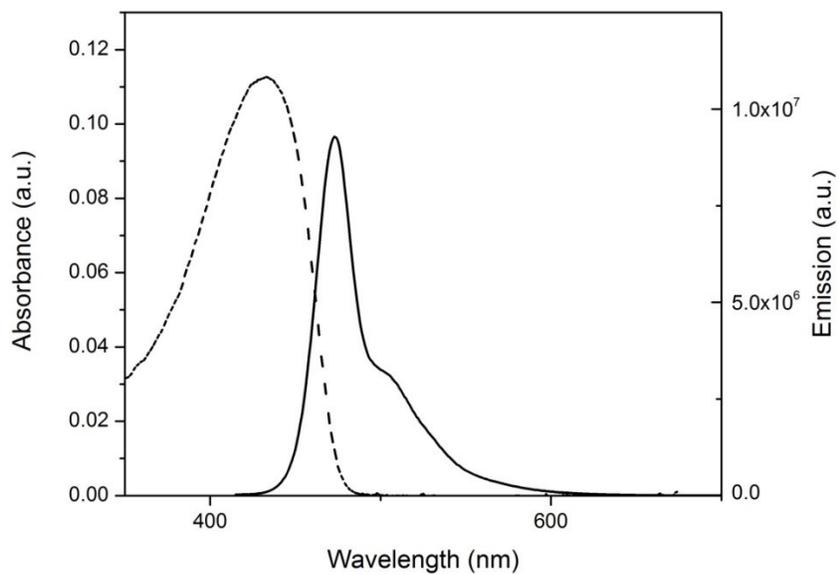


Figure S15. UV-vis and emission spectra of **PPE-2-Br** in DCM.

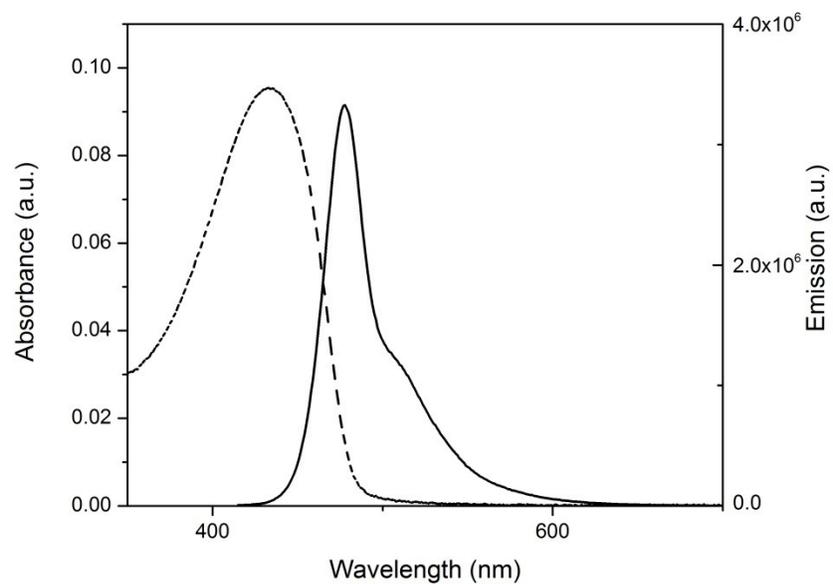


Figure S16. UV-vis and emission spectra of **PPE-2** in DMSO.

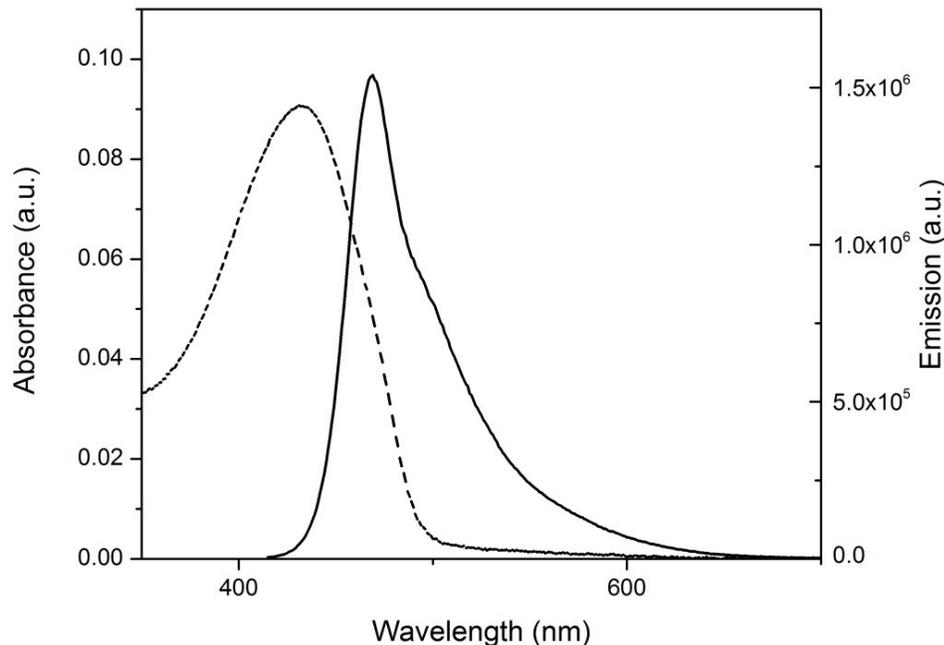


Figure S17. UV-vis and emission spectra of **CPN-2** 1% DMSO in water (v/v).

3.3 Synthesis of non-degradable PPE with low molecular weight.

PPE-3-Br. Using general procedure described above, the polymerization of monomer **M1** (104.0 mg, 0.138 mmol) and **M2** (50.0 mg, 0.110 mmol) in presence of Pd[(PPh₃)₂Cl₂] (9.3 mg, 0.013 mmol), and CuI (1.3 mg, 0.007 mmol). Resulting mixture was purified by precipitation in methanol (x3) Yield: 0.09 g (67.6%). An aliquot was redissolved in THF and molecular weight obtained and photo physical properties characterized in DCM.

PPE-3-Br: GPC: $M_w = 09.62$ kDa; $M_n = 06.96$ kDa; PDI = 1.38. UV-vis: $\lambda_{max} = 428$ nm; fluo $\lambda_{max} = 473$ nm; QY = 15% and $\epsilon = 15931.37$ M⁻¹ cm⁻¹(Per repeating Unit) in DCM. ¹H NMR (400 MHz, CDCl₃): δ 7.05 (br, 4H), 4.24 (br, 9H), 3.92 (br, 9H), 3.78 (br, 12H), 3.72 (br, 2H), 3.65 (br, 13H), 3.51 (8H), 3.44 (br, 4H), 3.35 (br, 9H).

Synthesis of PPE-3.

Triphenyl phosphine (90 mg, 1.02 mmol) was dissolved in chloroform and resulting solution was added to a stirred suspension of **PPE-3-Br** (33 mg, 0.102 mmol). The reaction was heated to 85°C for 12 h. The resulting mixture was purified by precipitation in ethyl acetate (x5). Yield: 26 mg (52.0%).

PPE-3: UV-vis: $\lambda_{max} = 428$ nm; fluo $\lambda_{max} = 476$ nm; QY = 6% and $\epsilon = 29777.78$ M⁻¹ cm⁻¹(Per repeating Unit) in DMSO. ¹H NMR (400 MHz, DMSO-d₆): δ 7.76 (br, 30H), 7.14 (br, 4H), 4.16

(br, 8H), 3.91 (br, 4H), 3.76 (br, 6H), 3.61 (br, 14H), 3.45 (br, 12H), 3.26 (br, 4H), 3.14 (br, 9H).
 ^{31}P NMR (161.9 MHz, DMSO- d_6): 24.84.

CPN-3: UV-vis: $\lambda_{\text{max}} = 427$ nm; fluo $\lambda_{\text{max}} = 468$ nm; QY = 5% were measured using 1% DMSO in water (v/v).

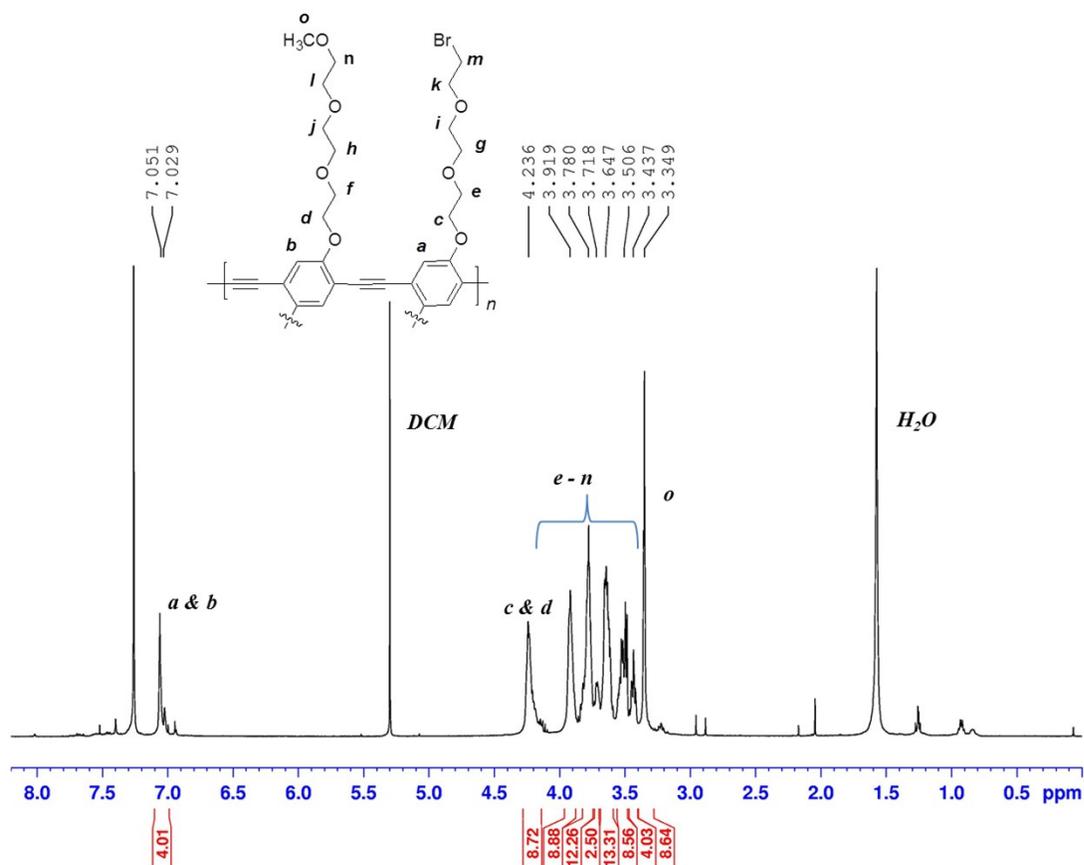


Figure S18. ^1H NMR of PPE-3-Br in CDCl_3 .

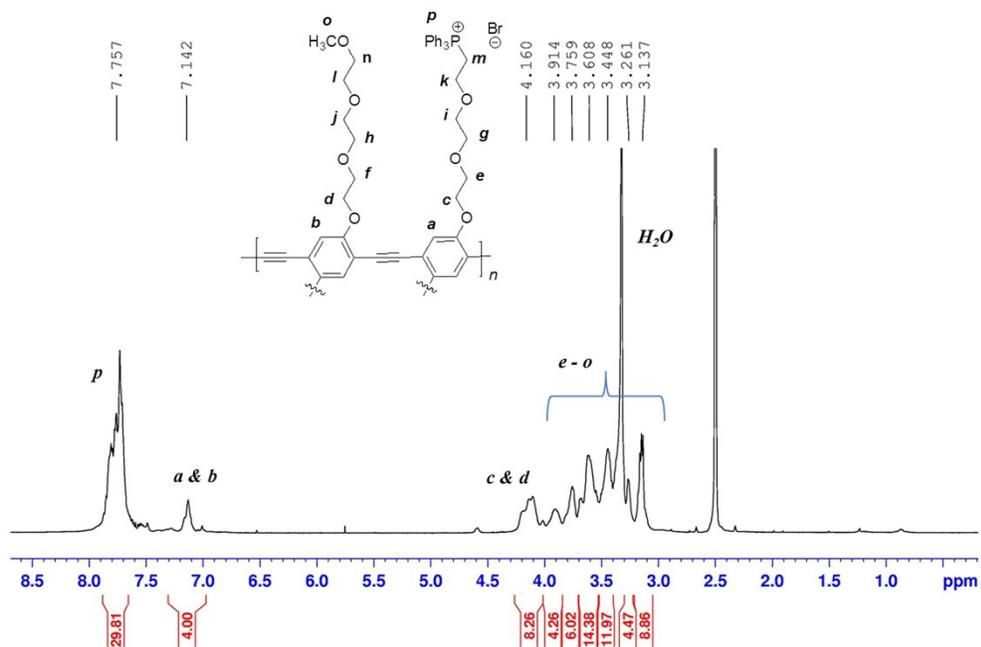


Figure S19. ^1H NMR of PPE-3 in DMSO-d_6 .

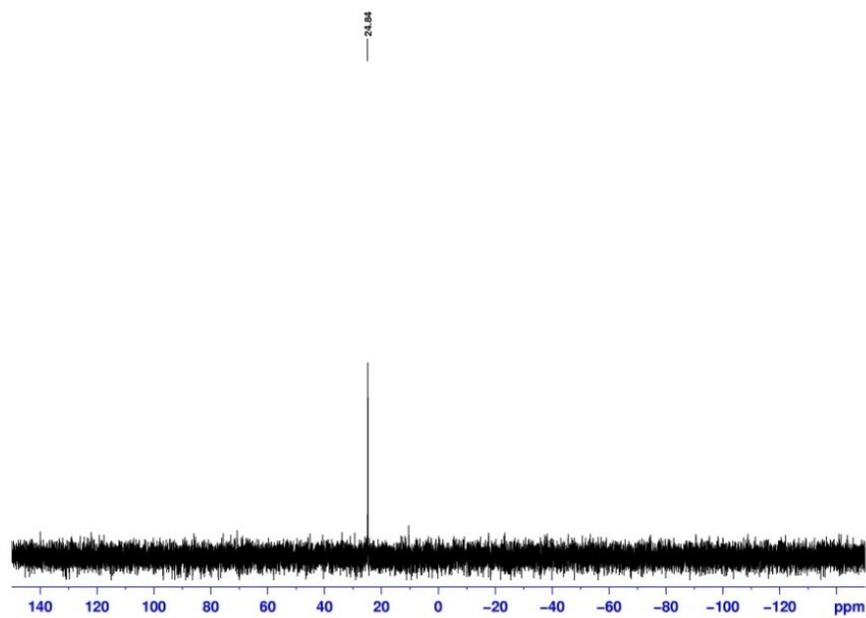


Figure S20. ^{31}P NMR of PPE-3 in DMSO-d_6 .

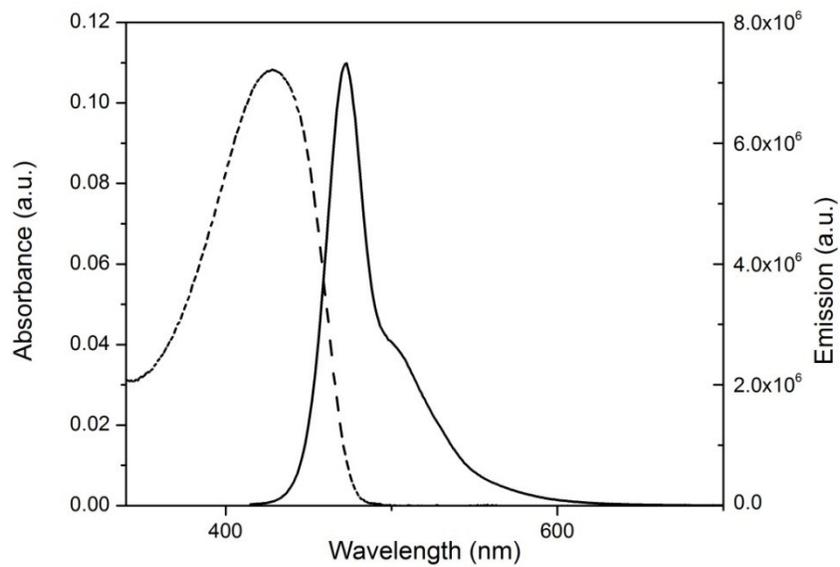


Figure S21. UV-vis and emission spectra of **PPE-3-Br** in DCM.

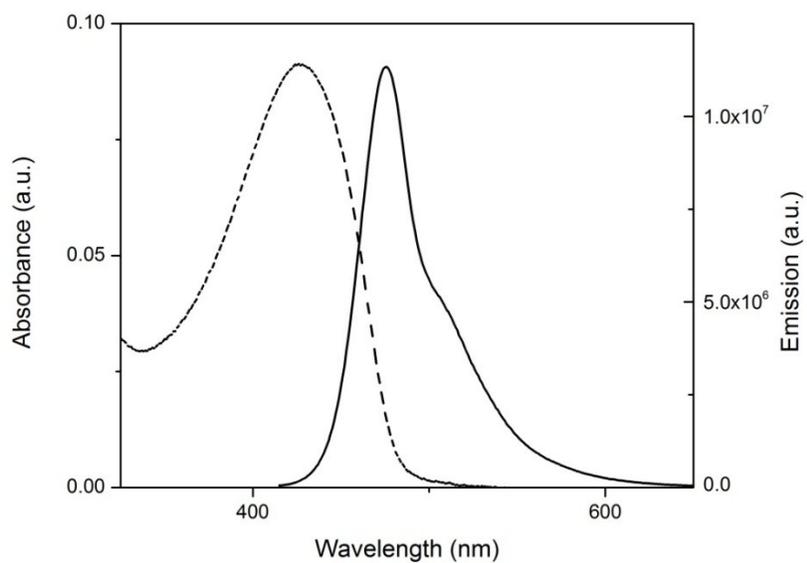


Figure S22. UV-vis and emission spectra of **PPE-3** in DMSO.

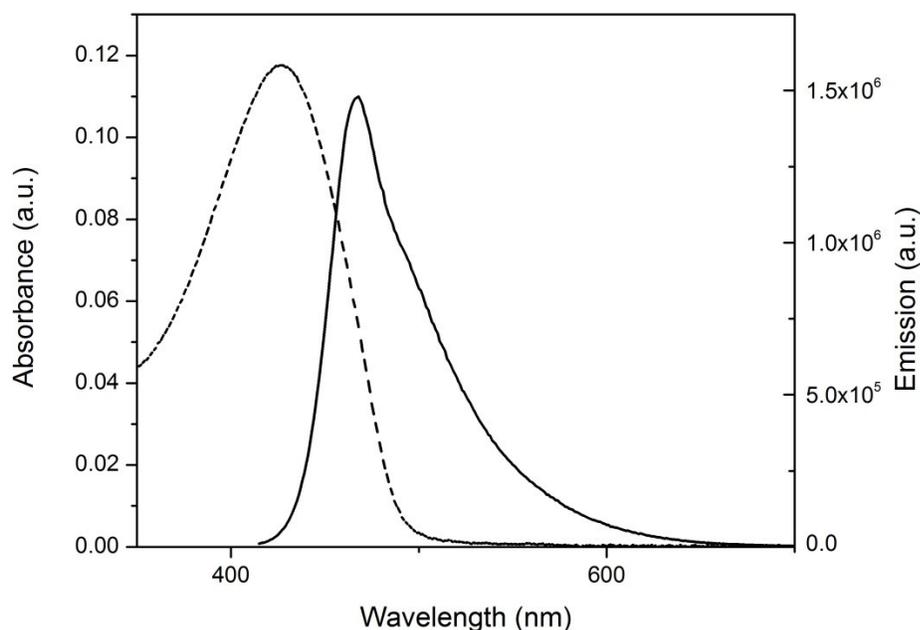


Figure S23. UV-vis and emission spectra of **CPN-3** 1% DMSO in water (v/v).

3.4 Particle formation and HA complexation.

Particles were prepared prior to each experiment from a concentrated polymer stock solution in DMSO by diluting with an appropriate volume of filtered (0.22 μm PTFE syringe filter) deionized water (18 Ω). Sodium hyaluronate (HA) was purchased from Lifecore (MW 40 KDa) and used as received. A stock solution was prepared by dissolving 2.0 mg of HA in 1 mL of deionized water. CPN/HA complexes were prepared by mixing CPN and HA at a molar ratio of 1 to 3 (based on polymer and disaccharide repeating unit) then diluting to the desired concentration with deionized water. CPN/HA complexes were allowed to incubate for 30 minutes prior to measurement.

3.5 Determination of hydrodynamic diameters of CPNs and CPN/HA complexes.

Nanoparticle tracking analysis (NTA) measurements were performed with a LM10 HS (NanoSight, Amesbury, United Kingdom), equipped with a sCMOS camera, sample chamber with a 488 nm blue laser, and Viton fluoroelastomer o-ring. The CPN samples were prepared from concentrated stock solutions of polymer in DMSO, at approximately 10 μM in a 0.5% DMSO (v/v) solution using deionized water (18 Ω) filtered through 0.22 μm polytetrafluoroethylene (PTFE) syringe filter. The samples were injected into the sample chamber with 1 mL sterile syringes (Restek Corporation, Pennsylvania, USA) until the liquid reached the tip of the nozzle. All measurements were performed at 25°C using a LM14C temperature controller (NanoSight, Amesbury, United Kingdom). Each sample was measured three times.

Table 1. Hydrodynamic diameters and zeta potential of CPNs.

Entry	Hydrodynamic diameter (nm)	Zeta potential (mV)
CPN-1	139.9 ± 3.0	+ 23.5 ± 1.84
CPN-1/HA	191.8 ± 2.6	- 44.4 ± 1.01
CPN-2	152.7 ± 6.7	+ 29.2 ± 3.56
CPN-2/HA	223.1 ± 6.9	- 42.7 ± 0.83
CPN-3	158.8 ± 16.4	+ 20.1 ± 5.87

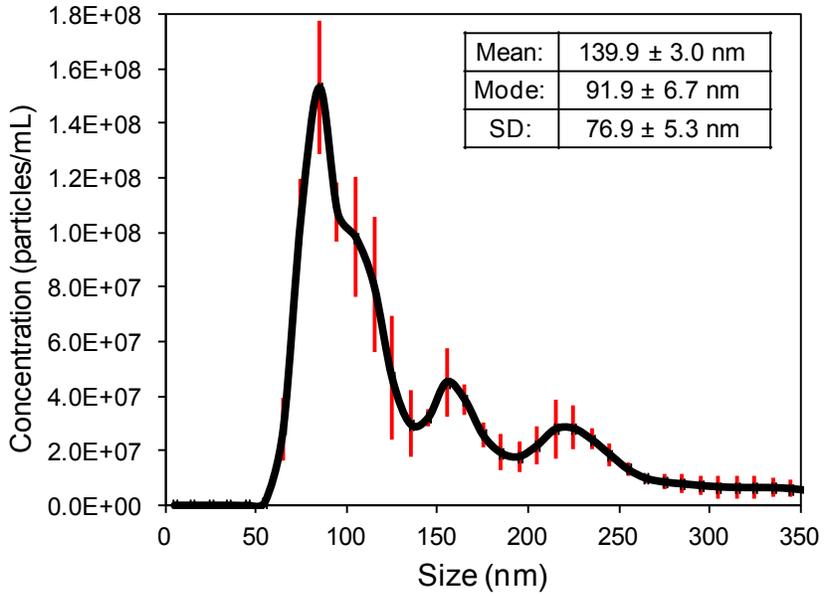


Figure S24. NTA for CPN-1.

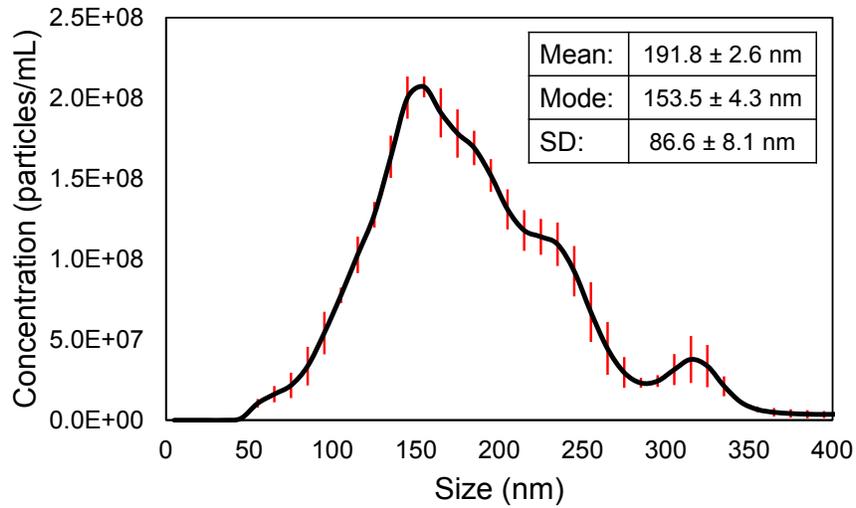


Figure S25. NTA for CPN-1/HA.

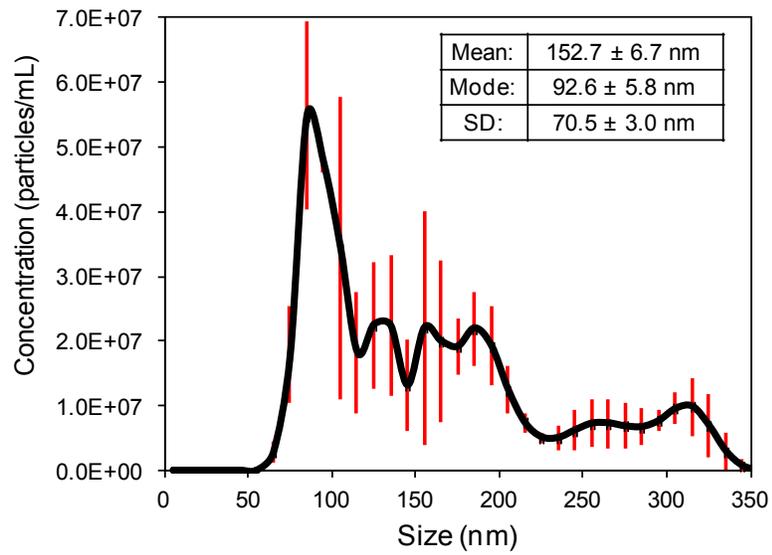


Figure S26. NTA for CPN-2.

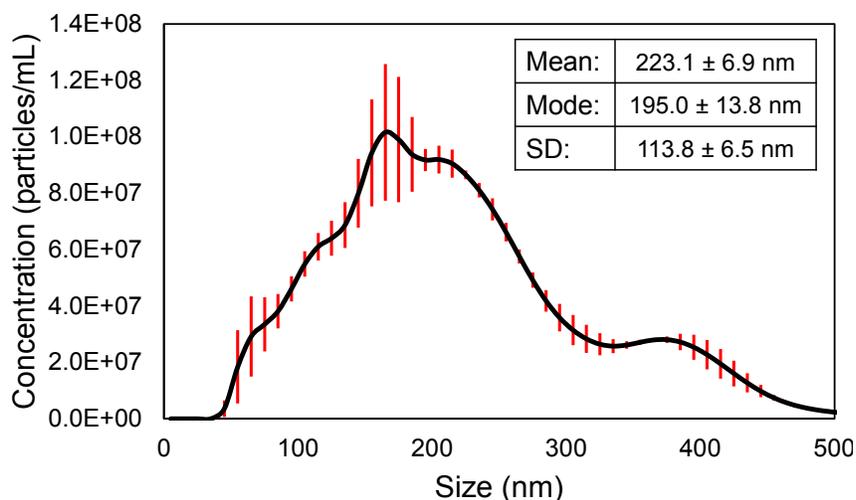


Figure S27. NTA for CPN-2/HA.

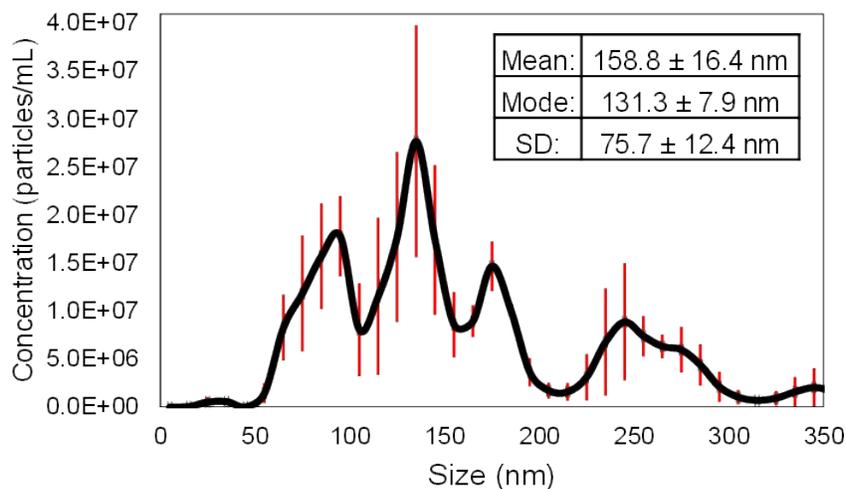


Figure S28. NTA for CPN-3.

3.6 Determination of zeta potentials of CPNs and CPN/HA complexes

Dynamic light scattering (DLS) measurements were performed by Zetasizer nano-ZS (Zen 3600, Malvern Instruments Ltd.) using a folded capillary cell (Catalog # DTS1060), at room temperature. The CPN samples were prepared from concentrated stock solutions of polymer dissolved in DMSO, at approximately 0.1 mM in a 5% DMSO (v/v) solution using deionized water (18 Ω) filtered through 0.22 μm PTFE syringe filter. Each sample was measured six times.

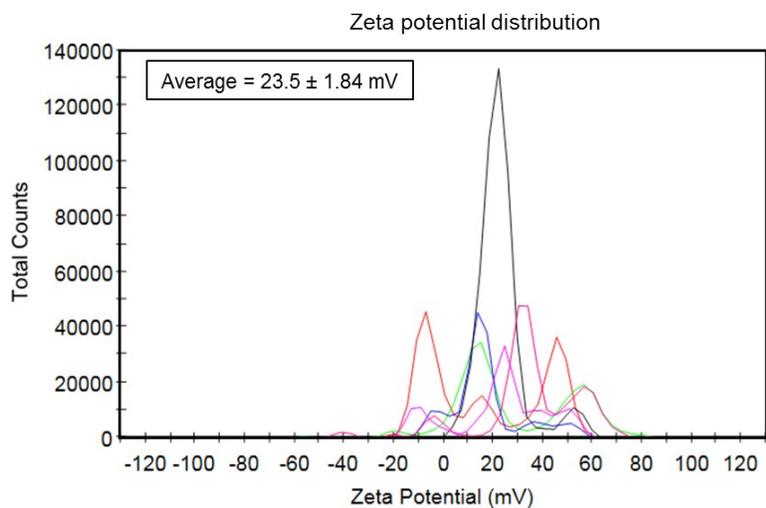


Figure S29. Zeta potential of CPN-1.

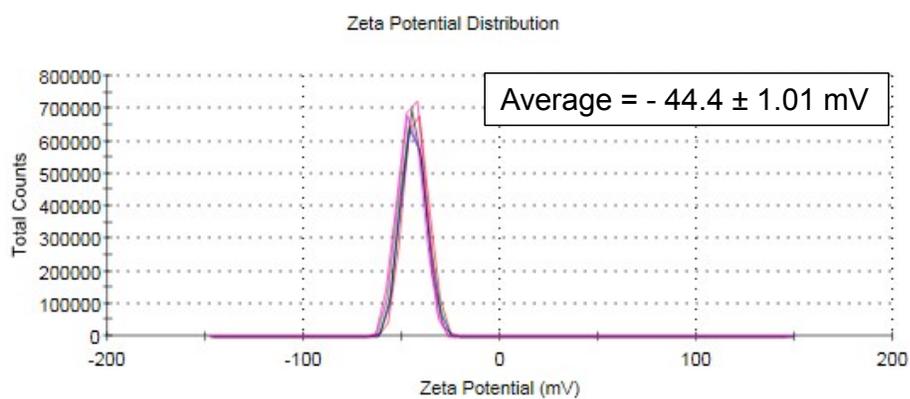


Figure S30. Zeta potential of CPN-1/HA.

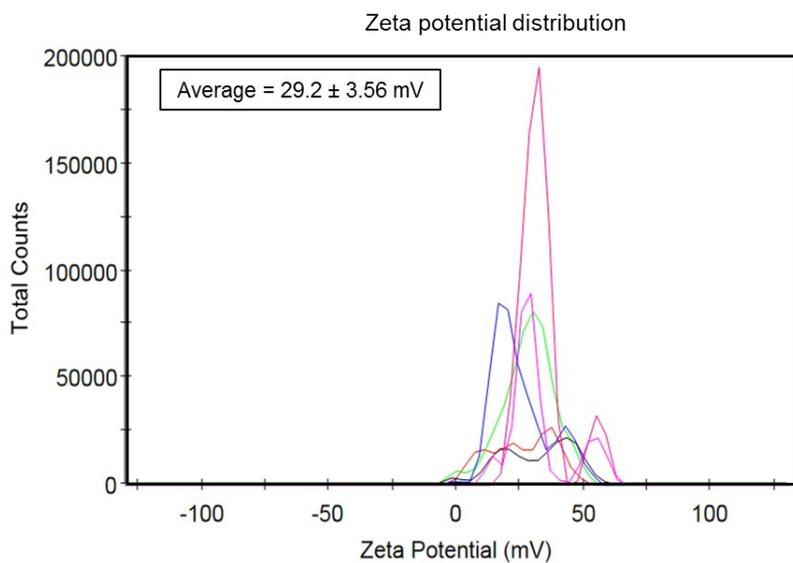


Figure S31. Zeta potential of CPN-2.

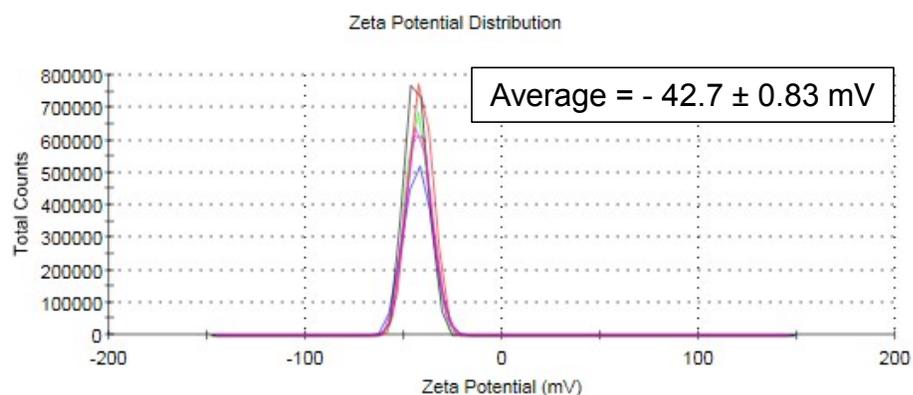


Figure S32. Zeta potential of CPN-2/HA.

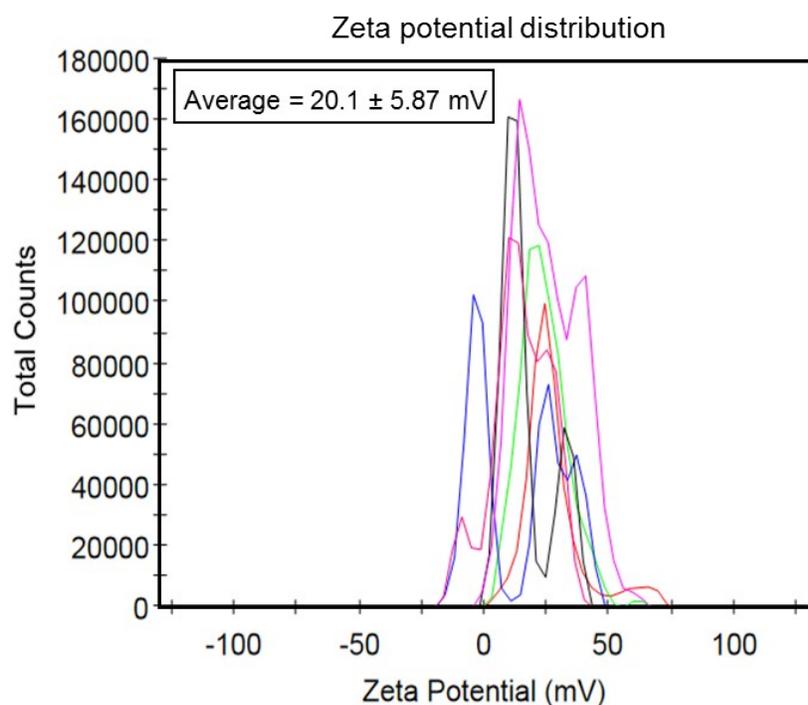


Figure S33. Zeta potential of CPN-3.

3.7 Degradation of CPN-1.

To test degradation under intracellular conditions, CPN-1 (5 μ M in H₂O) was treated with excess glutathione (GSH) at 10 mM. The UV-vis and fluorescence emission spectra (see Figure S34) were recorded for CPN-1 only, upon immediate addition of GSH, and after 18 h at 37°C with GSH.

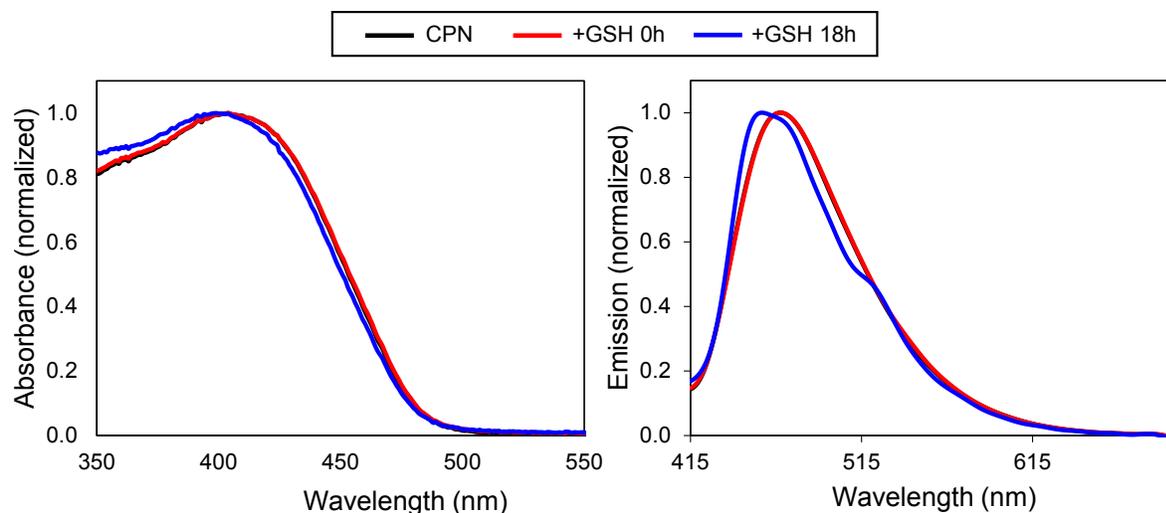


Figure S34. Absorption (left) and emission (right) spectra of CPN-1 treated with 10 mM glutathione (GSH) for 0 and 18 h.

4.0 General Cell Culture.

HeLa cells (human cervical cancer, purchased from ATCC) were seeded into a 100 x 20 mm sterile tissue culture dish (#353003 BD Falcon, Durham, NC, USA). Then, they were cultured in complete media: Dulbecco's minimum essential medium (DMEM)/high glucose (10 mL, HyClone, SH3024301) medium containing 10% fetal bovine serum (FBS), 100 U/mL penicillin and 100 µg/mL streptomycin (P/S) for 24 h under 5% CO₂ at 37°C. Then, they were subcultured every 48 h. pHRhodo Dextran 10 kDa (#P10361 Molecular probes, Life technologies, New York, USA), MitoTracker Deep Red (#M22425 Molecular probes, Life technologies, New York, USA) and BODIPY-TR C5-ceramide-BSA complex (#B34400 Molecular probes, Life technologies, New York, USA) were used for endosome, mitochondria, and Golgi apparatus staining, respectively. The isolation of mitochondrial and cytosolic fractions of HeLa cells was performed using an Eppendorf Centrifuge 5430R, Mitochondria Isolation Kit for cultured cells (#89874, Thermo Fisher Scientific), and Pierce BCA Protein Assay Kit (#23227, Thermo Fisher Scientific). Pharmacological endocytosis inhibitors such as chlorpromazine hydrochloride (#ALX-270-171-G005, Enzo Life Sciences, Inc.), genistein (#AC32827-1000, Acros Organics), methyl-β-cyclodextrin (#377110050, Acros Organics), LY2994002 (#70920, Caymen Chemical), and cytochalasin D (#BML-T109-0001, Enzo Life Sciences, Inc.) were purchased from Fisher Scientific.

4.1 Toxicity Assay

HeLa cells (ca. 10,000 cells per well), in 200 µL of complete medium, were seeded into a 96-well plate and cultured for one day in a 5% CO₂ incubator at 37 °C. 4 mM of CPN-1, CPN-2 and CPN-3 stock solutions were prepared by dissolving dried powder in DMSO. 2 mM, 1 mM, 0.5 mM, and 0.1 mM substock solutions were made by diluting with DMSO. Final concentrations of 40 µM, 20

μM , 10 μM , 5 μM , and 1 μM of CPNs were added into complete media by dilution with CPN-1, CPN-2, and CPN-3 stock solutions and then incubated for 18 h. To measure toxicity, 10 μL of MTT solution (5 mg mL^{-1} in PBS, CALBIOCHEM, Germany) and 90 μL of complete medium were then added into each well, and the plate was further incubated for 4 h at 37 °C. After the formed MTT formazan crystals were dissolved in dimethyl sulfoxide (100 μL), the absorbance intensity was measured by a microplate well reader (infinite M1000 PRO, TECAN, Switzerland) at 540 nm. Relative cell viability (%) as a function of CPN concentration was expressed as a percentage relative to the untreated control cells. All measurements were performed in triplicate and standard deviation was included in the error bar (see Figure S30).

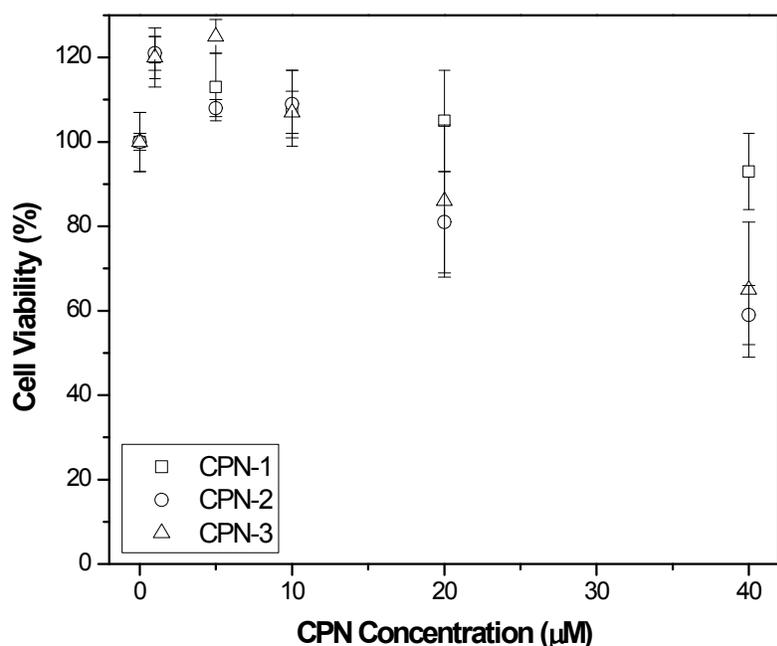


Figure S35. Cellular toxicity of CPN-1, CPN-2, and CPN-3.

4.2 Microscopic imaging, co-localization, and time-course subcellular localization.

10,000 HeLa cells were seeded into a glass-bottomed eight-well chamber slide (Lab-Tek Thermo Scientific) and cultured in complete media (400 μL) for 24 h under 5% CO_2 at 37 °C. 80 μL of 20 μM CPNs in water was added to the culture medium directly, and the cells were further cultured overnight (final CPN concentration: 4 μM). For endosome staining, pHRhodo Dextran 10kDa (5 μM) was incubated for 30 min at 37°C. For Golgi apparatus staining, BODIPY-TR C5 - ceramide-BSA complex (final 10 μM) was incubated for 30 min at 4°C. For mitochondria staining, MitoTracker Deep Red (100 nM) was incubated for 30 min at 37°C. After washing with fresh medium, the cells were further incubated for 15 min at 37 °C. A 1 μL aliquot of Hoechst (5 $\mu\text{g}/\text{mL}$) was added to the culture medium and incubated with the cells for 10 min at 37°C, and washed two times with phosphate buffered saline (PBS). The cells were fixed with 4% paraformaldehyde for 10 min. Fluorescent images of the cells were obtained using a DeltaVision Elite Microscope

System (Applied Precision, Issaquah, Washington, USA) equipped with bandpass filters such as blue (410–460 nm, Hoechst), green (500–550 nm, CPNs), and red (595–635nm, Golgi apparatus, endosome, and mitochondria) using a 60X oil immersion lens (NA 1.42) and n = 1.520 immersion oil. Top and bottom of the chosen cells was identified, and a Z-stack plot was imaged for each channel. Z-stack microscope images of each sample was obtained as described above. Colocalization analysis was conducted for three independent cells per polymer and per organelle (Golgi apparatus, endosome, and mitochondria). Colocalization analysis was conducted using the microscope software (Softworx 5.0 application, Applied Precision, Issaquah, Washington, USA). Region of interest (ROI) was selected to contain all of the cell. Pearson's Correlation Coefficient (PCC) was used to determine colocalization. Negative control of colocalization was performed by analysis of blue and green channels staining the nucleus and CPN, respectively. Three independent images of an entire cell were selected and analyzed to increase analysis objectivity.

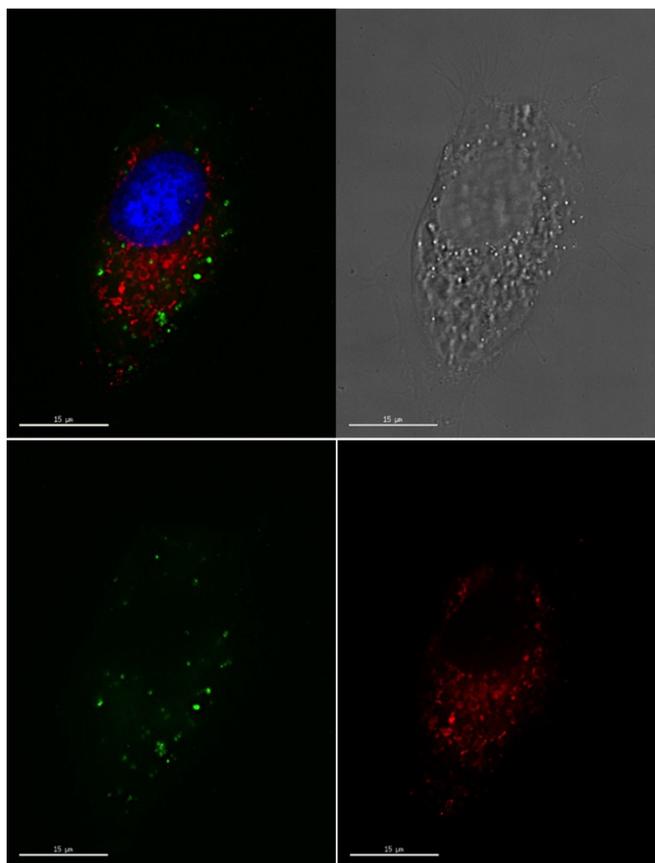


Figure S36. Microscopic images of HeLa cells incubated with CPN-3 for 18 h followed by mitochondrial (red) and nuclear (blue) staining. The scale bar is 15 µm.

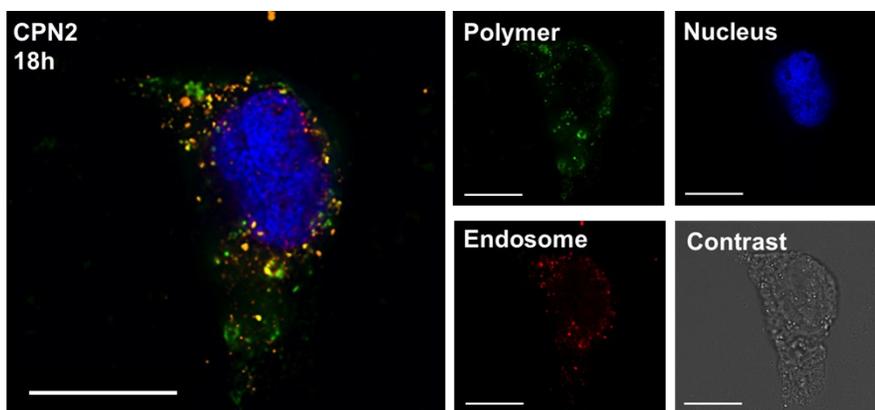


Figure S37. Microscopic images of HeLa cells incubated with **CPN-2** for 18 h followed by endosome (red) and nuclear (blue) staining. The scale bar is 20 μm .

4.3 Confocal microscopic imaging, CPN/HA co-localization, and concentration-dependent co-localization.

HeLa cells were seeded into a 12-well plate ($\sim 50,000/\text{well}$) with glass coverslip (#1254584, Fisher Scientific) one day prior to CPN treatment and cultured in complete media (500 μL) for 24 h under 5% CO_2 at 37°C. After cell attachment, fresh complete media (500 μL) containing 50 μL of 2 μM or 20 μM CPN in H_2O (final CPN concentration: 0.2 μM or 2 μM , see Figure S37) was added and cultured for 18 h. For CPN/HA, fresh complete media (500 μL) containing a premixed solution of 50 μL of 2 μM CPN in H_2O (final CPN concentration: 0.2 μM) and 15 μL 20 μM HA was added and cultured for 18 h. After incubation, cells were washed with 1X PBS and stained with MitoTracker Deep Red (50 nM) in DMEM only (no FBS or P/S) for 30 min at 37°C. After washing with fresh DMEM for 15 min at 37°C, cells were fixed with 4% paraformaldehyde for 10 min at room temperature. After washing with 1X PBS, a 1 μL aliquot of Hoechst (5 $\mu\text{g}/\text{mL}$) was added to 199 μL PBS and incubated with the cells for 10 min at room temperature. Nuclear staining is not shown in Figure S37 due to non-specific binding of aggregated CPNs at high concentration. Cells were then washed three times with 1X PBS and coverslips were mounted on microscope slides (#1125441, Fisher Scientific) using a 1:1 glycerol/PBS mounting medium. Fluorescent images of the cells were obtained using an Olympus FluorView FV1200 confocal microscope (Melville, NY USA) equipped with bandpass filters such as blue (417–477 nm, nucleus), green (513–556 nm, CPNs), and deep red (672–712 nm, mitochondria) using a 60X oil immersion lens (NA 1.35) and $n = 1.519$ immersion oil. The top and bottom of the samples were identified, and a Z-stack plot was imaged for each channel. Co-localization analysis of the green (CPN) and red (mitochondria) channels was conducted for three independent images using ImageJ software (version 1.50b, U. S. National Institute of Health, Bethesda, Maryland, USA). PCC scores were calculated to determine co-localization by setting pixel threshold for the entire image per channel and per sample in order to exclude noise and background signal.⁴

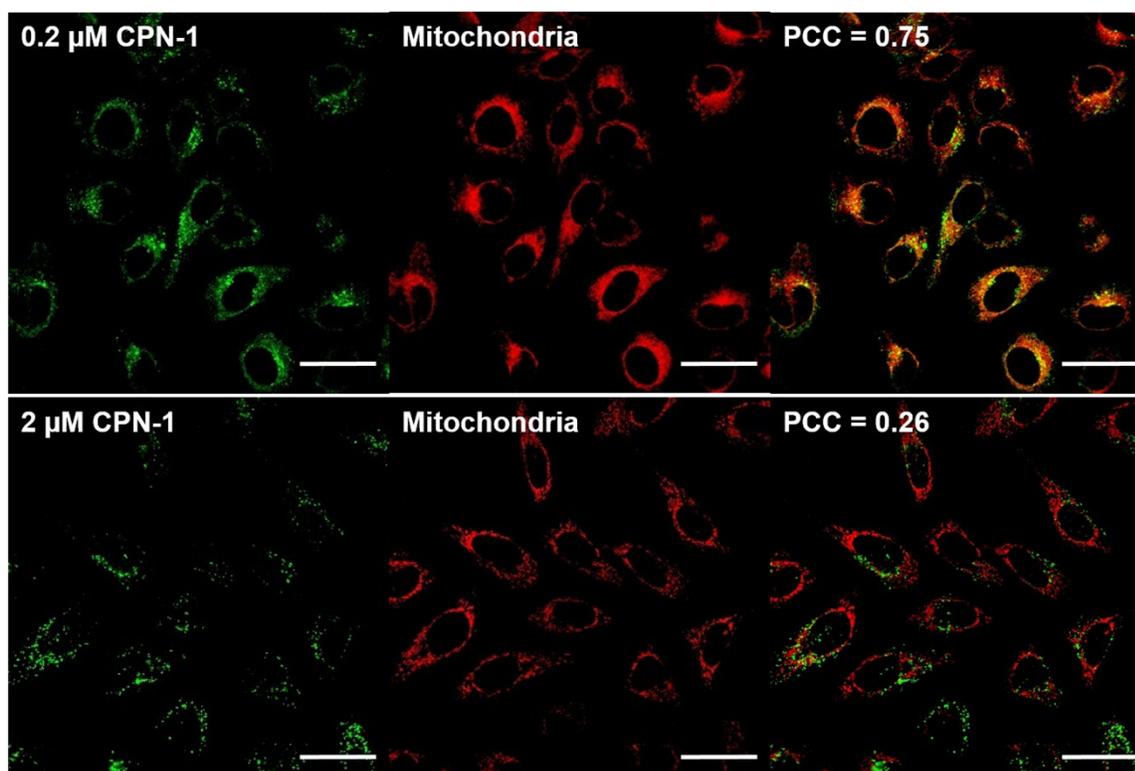


Figure S38. Confocal microscopic images of HeLa cells incubated with CPN-1 0.2 μM (top row) and 2 μM (bottom row) for 18 h followed by mitochondria (red) staining.

4.4 Isolation of Mitochondrial and cytosolic fractions of HeLa cells.

HeLa cells (ca. 10,000,000) were seeded into a 100 x 20 mm style sterile tissue culture dish (#353003 BD Falcon, Durham, NC, USA) with 10 mL of complete medium. After 24 h incubation at 37 °C, cells were treated with 5 μM of CPN-1 and CPN-2, respectively, for 24 h. After washing with PBS three times, the mitochondrial and the cytosolic fractions were isolated using a commercial kit (Mitochondria Isolation Kit for cultured cells #89874, Thermo Fisher Scientific). Briefly, HeLa cells were isolated by scraping and washed with PBS. The kit reagent A was added followed by incubating on ice for 2 min. The kit reagent B was added followed by vortexing and incubation on ice. After 5 min of incubation, the kit reagent C was added and the cells were centrifuged (700 x g at 4 °C). The pellet containing nuclei and cellular debris was discarded and the supernatant was centrifuged (12,000 x g at 4 °C). The supernatant containing the cytosolic fraction was removed and the pellet composed of mitochondria was washed with the kit reagent C. The pure mitochondria fractions were isolated by centrifugation (12,000 x g at 4 °C). The mitochondrial fraction and the cytosolic fraction were diluted to 200 μL 100% DMSO and 200 μL of 50% DMSO, respectively. Then the total fluorescence intensities of the mitochondrial and the cytosolic fractions resulting from the excitation at 455 nm were measured, normalized by the amount protein (μg) of the fraction, and determined by BCA protein assay (Pierce BCA Protein Assay Kit #23227, Thermo Fisher Scientific).

4.5 Flow Cytometry

HeLa cells incubated with CPNs were treated with trypan blue (Amresco Inc.) before flow cytometry measurements (Accuri C6, USA). HeLa cells incubated with 10 μ M of CPN for 4 h were treated with trypan blue (200 μ M) for 10 min. After washing with 1X PBS buffer three times, cells were detached by trypsin/EDTA and fixed with 4 % paraformaldehyde. Post-cell treatment with trypan blue was conducted for all flow cytometry analyses for entry kinetics and mechanisms.

4.6 Endocytosis Inhibition Assay

For endocytosis inhibition studies, HeLa cells were seeded into a 6-well plate (\sim 200,000/well) one day before CPN treatment. Cells were then treated without (control) or in the presence of pharmaceutical inhibitors chlorpromazine (24.0 μ M), genistein (0.21 μ M), methyl- β -cyclodextrin (1.00 μ M), LY294002 (0.12 μ M), or cytochalasin D (0.04 μ M) for 30 min before CPN treatment. After CPN treatment, cells were washed with 1X PBS three times followed by trypan blue treatment (0.20 μ M) for 10 m. The detached cells were fixed with 4 % paraformaldehyde in 1x PBS for 10 m and resuspended in a flow cytometry buffer (1X PBS containing 5 % bovine serum albumin and 0.02 % sodium azide). 10,000 events per measurement were counted and mean fluorescence intensity of CPN (FL2 channel, 518-548 nm wavelength range) was measured. Averaged mean fluorescent intensity was calculated using three independent sample sets.

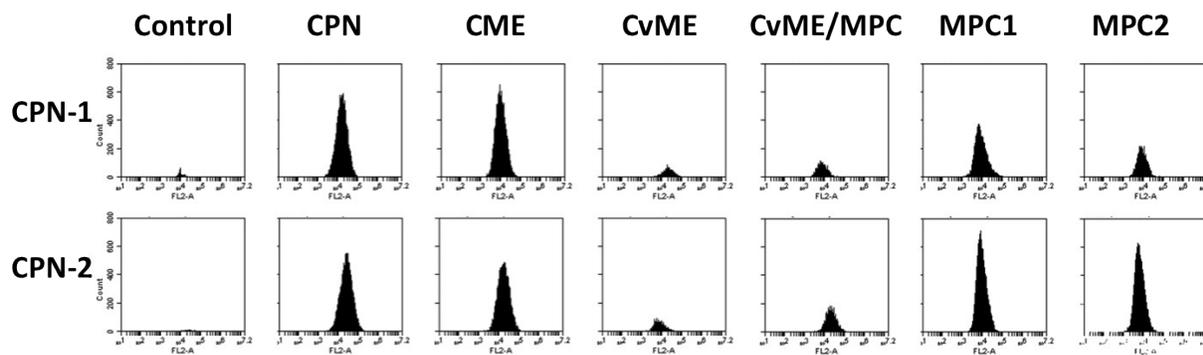


Figure S39. Fluorescent intensity plots obtained at the FL2 channel (518-548 nm).

5.0 References

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