SUPPORTING INFORMATION

Effects of Side-Chain Isomerism on Polymer-based Non-Covalent Protein Delivery

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8.2 Polymer Synthesis

1. Materials and Methods

Chemicals, solvents, and cell culture materials were purchased from Fisher Scientific and used without further purification. PULSin was purchased from PolyPlus. Bovine serum albumin (BSA, 67 kDa, pI 4.7), immunoglobulin G from human serum (IgG, 150 kDa, pI 7.3), red algae-phycoerythrin (R-PE, 240 kDa, pI 4.3), Beta-Galactosidase (B-Gal, 465 kDa, pI 4.6), fluorescein isothiocyanate labeled BSA and IgG (FITC-BSA and FITC-IgG), and Phospho-AKT1 (Ser473) monoclonal antibody (Anti-pAkt) were purchased from Fisher Scientific. Deuterated solvents were purchased from Cambridge Isotope Laboratories. HeLa, bEnd.3, and Caco-2 cells were purchased from ATCC. The number average molecular weight (M_n), weighted average molecular weight (M_w), and polydispersity index (PDI) of polymers were determined by gel permeation chromatography (GPC) against polystyrene standards using a Shimadzu high- performance liquid chromatography (HPLC) system fitted with PLgel 5μm MIXED-D columns and SPD-20A ultraviolet-visible (UV-vis) detector at a flow rate of 1.0 mL/min. Nuclear magnetic resonance (NMR) spectra were recorded on a 400 MHz Bruker Avance NMR spectrometer. Hydrodynamic diameters were obtained using an LM10 HS (NanoSight, Amesbury, United Kingdom), equipped with an sCMOS camera, a sample chamber with Viton fluoroelastomer O-ring, and a 488 nm blue laser. Zeta potentials were determined by using a Zetasizer Nano-Zs (Zen 3600, Malvern Instruments) using a folded capillary cell (DTS1070). Fluorescence emission spectra were recorded using a FluoroLog-3 Spectrofluorometer (Horiba). Absorbance measurements were recorded on a Tecan Infinite M1000 Pro microplate well reader at 570 nm. Flow cytometry experiments were done using a BD FACSCelesta system and data was analyzed using FlowJo VX. Confocal laser scanning microscopy images were collected using a Nikon A1R microscope with a 60x objective with oil immersion. DAPI, FITC, and TRITC channels were used for fluorescence detection. Brightfield images were obtained using Invitrogen EVOS M7000.

2. Physical Characterization of PNs and PN/Protein Complexes

2.1 Molecular Weight Determination

Aliquots of polymer solutions in tetrahydrofuran (THF) or dichloromethane (DCM) were diluted in 1 mL of HPLC-grade THF and filtered through a 0.45 μm polytetrafluoroethylene (PTFE) syringe filter prior to GPC analysis.

2.2 Molecular Dynamic Simulations

The structures of the Ph-CG and Ph-CG' polymers were generated from the ligand reader interface of the CHARMM-GUI.[1] Simulation systems were prepared for the four compounds using the Solution Builder plugin of the CHARMM-GUI.[2] The compounds were solvated in TIP3 water and 150 mM of KCl.[3] A GPU version of NAMD 2.14 was used to perform all-atom molecular dynamics (MD) simulations with CHARMM36 force field at 303.15 K temperature.[4] The systems were minimized for 10,000 steps, followed by 0.25 ns equilibration with position restraints. Finally, 100 ns unconstrained production run was performed with a timestep of 2 fs for each system. The covalent bonds involving hydrogen atoms were restrained with SHAKE algorithm and the long-range electrostatic interactions were treated with particle mesh Ewald method. Temperature was controlled by Langevin temperature coupling with 1 ps of friction coefficient and the pressure was controlled by Langevin piston. The visualization and data analysis were performed using VMD.1.9.3.

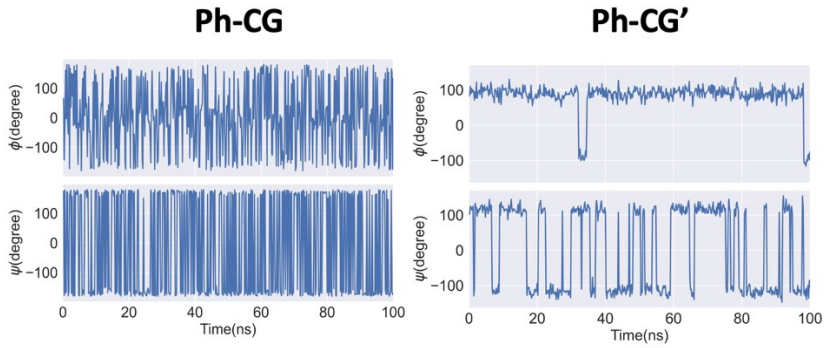


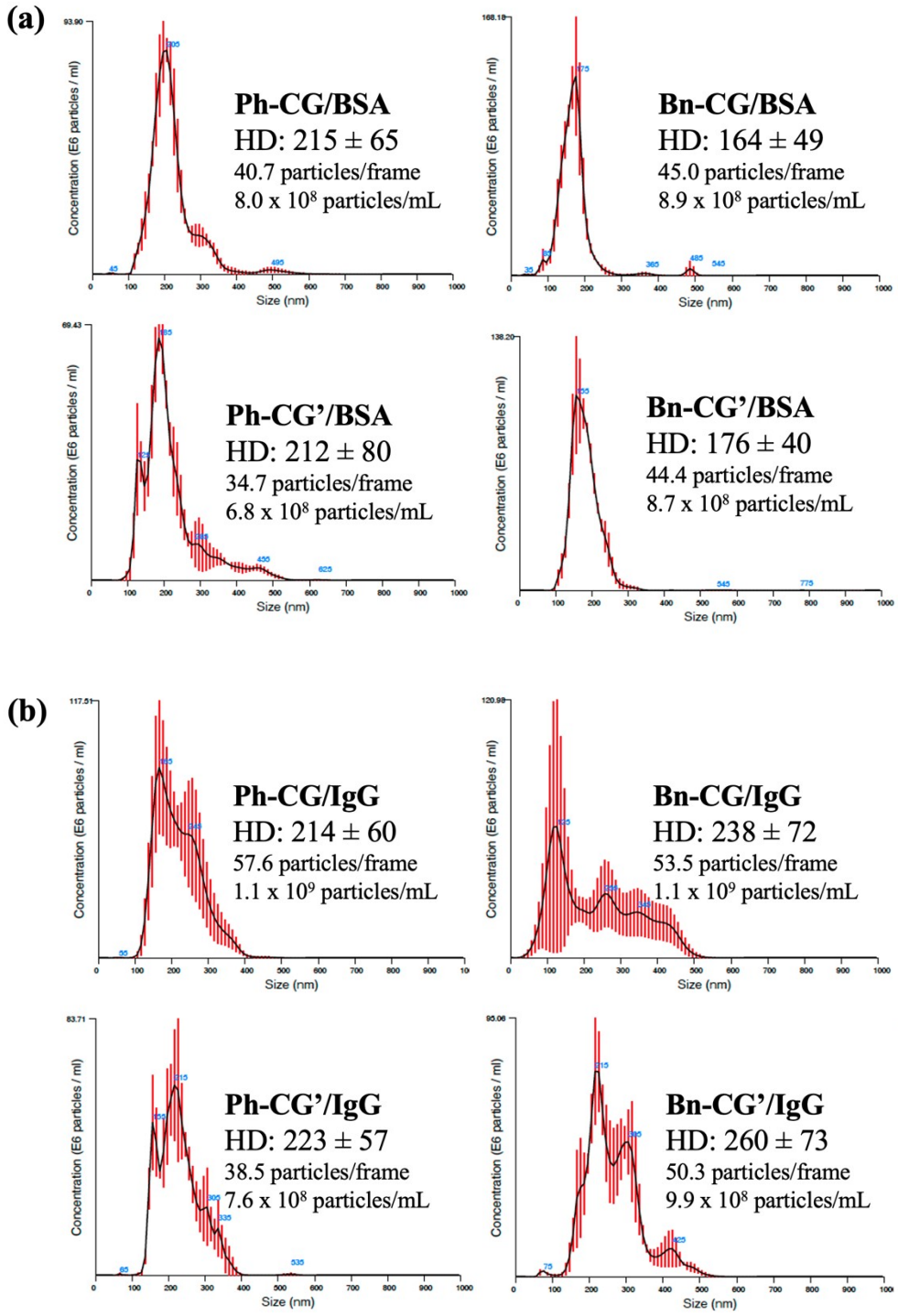
Figure S1. Fluctuations in dihedral angles ϕ and ψ as a function of simulation time.

2.3 Measurement of Hydrodynamic Diameters and Zeta Potential

Nanoparticle tracking analysis (NTA) was used to determine the hydrodynamic diameter (HD) of PN/protein complexes. Briefly, PN stock solutions were prepared by dissolving dried PN powders in DMSO and diluting to final concentrations of 1 mM in DMSO. Adequate volmes of PN stock solutions were diluted in water (20% v/v) and mixed with equal volumes of BSA solution for a final complex volume of 100 μL. Complexes were incubated for 30 min at room temperature before a ten-fold dilution in PBS to a final volume of 1 mL (final PN/protein concentration was 10 μM and 10 μg/mL, respectively). 1 mL of this solution was then injected into the NTA chamber and videos of the scattering particles was recorded for 30 seconds. The software identified each individual particle and tracked its motion, relating the particle displacement as a function of Brownian motion, which relates to the particle size through the Stokes-Einstein equation. The concentrations of samples were chosen to meet the manufacturers' recommendation of 20-100 particles per frame and a concentration of 10^7-10^9 particles/mL. All measurements were performed in triplicate at 25 °C. HD plots are listed below for PN/BSA complexes, demonstrating the formation of monodisperse nanoparticles. Values given are the mean HD of three readings +/- mean standard deviation (SD). In this study, SD is defined as the arithmetic value calculated with the sizes of all of the particles analyzed by the software. Zeta Potential was acquired by preparing samples in the same way as for NTA prior to analysis.

Table S1. Summary of hydrodynamic diameters (HD) for PN/protein complexes analyzed by NTA

	Hydrodynamic diameter HD (nm)			
	BSA	**IgG**	**R-PE**	**B-Gal**
Ph-CG	215 ± 65	214 ± 60	194 ± 49	198 ± 43
Bn-CG	164 ± 49	238 ± 72	159 ± 55	173 ± 47
Ph-CG'	212 ± 80	223 ± 57	212 ± 55	169 ± 40
Bn-CG'	176 ± 40	260 ± 73	210 ± 46	291 ± 93


(a)
Ph-CG/BSA
HD: 215 ± 65
40.7 particles/frame
8.0 x 10^8 particles/mL
Bn-CG/BSA
HD: 164 ± 49
45.0 particles/frame
8.9 x 10^8 particles/mL
Ph-CG'/BSA
HD: 212 ± 80
34.7 particles/frame
6.8 x 10^8 particles/mL
Bn-CG'/BSA
HD: 176 ± 40
44.4 particles/frame
8.7 x 10^8 particles/mL
(b)
Ph-CG/IgG
HD: 214 ± 60
57.6 particles/frame
1.1 x 10^9 particles/mL
Bn-CG/IgG
HD: 238 ± 72
53.5 particles/frame
1.1 x 10^9 particles/mL
Ph-CG'/IgG
HD: 223 ± 57
38.5 particles/frame
7.6 x 10^8 particles/mL
Bn-CG'/IgG
HD: 260 ± 73
50.3 particles/frame
9.9 x 10^8 particles/mL
Concentration (E6 particles / ml)
Size (nm)

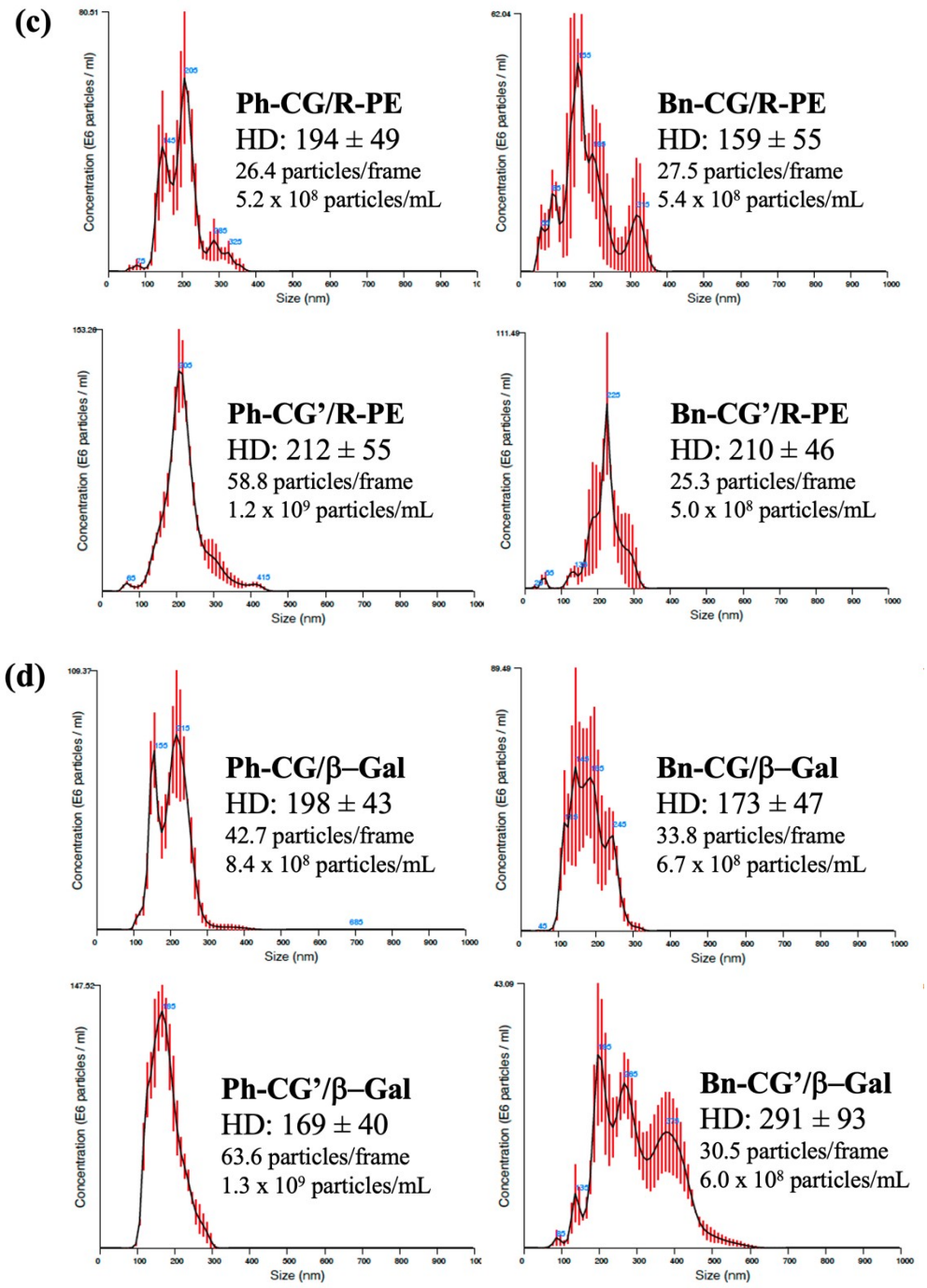


Figure S2. HD plots for PN/protein complexes for BSA (a), IgG (b), R-PE (c), and β-Gal (d), demonstrating the formation of relatively monodisperse nanoparticles after complexation.

2.4 pKa Determination

pKa was determined for the different PNs using previously described methods.[5] Briefly, a 2 mM polymer solution in 1 mL of acidified (pH ~3) 100 mM NaCl solution was preared and titrated from pH ~3 to 11 with 5 µL increments of a 25 mM KOH solution. For the titration, the pH was determined using a Mettler Toledo InLab Ultra-Micro pH Probe. pKa value for each polymer was determined by plotting the ΔpH/volume of KOH and identifying the largest ΔpH. For polymers with two maxima, the volume of the median point between the two maxima was chosen as the point where pH = pKa.

2.5 Dissociation Constant Determination

PN stock solutions were prepared by dissolving dried PN powders in DMSO and diluting to final concentrations of 1 mM in DMSO. 1 mM stock solutions were serially diluted in DMSO to a final concentration of 3.91 µM. Adequate volumes of each PN stock solution was diluted in water (20% v/v) and mixed with equal volumes of FP solution for a final complex volume of 50 µL. Complexes were incubated for 30 min at room temperature before a ten-fold dilution in PBS to a final volume of 500 µL (final concentration of BSA, IgG, and R-PE was 0.5 µg/mL, 5.0 µg/mL and 0.5 µg/mL, respectively). Final concentration of PNs ranged from 10 µM to 0 µM. The solutions were centrifuged at 22,000 x g for 10 min. The supernatant was collected in a cuvette and the fluorescence intensity of FPs in the supernatant was measured at their emission λ_{max}. The fluorescence intensity of each supernatant solution was related to 0 µM of PN yielding relative fluorescence curves as shown below (Figure S4). The relative fluorescence plots were converted to fractional saturation plots using equation 1 below:

$$Fractional\ Saturation\ (y) = \frac{F_P - F_0}{F_{sat} - F_0} \qquad (1)$$

where F_0, F_P, and F_{sat} were the relative emission intensities of FP only, free FP in supernatant at the various concentrations tested, and free FP at saturation. The dissociation constant was determined by equation 2 below:

$$y = \frac{(P + c + K_d) - \sqrt{(P + c + K_d)^2 - 4Pc}}{4c} \qquad (2)$$

where y is the fractional saturation plot obtained with equation 1, P is the polymer concentration (x-axis) and c is the constant FP concentration. K_d was determined using the non-linear curve fitting module of Origin 8.5 and equation 2. All R^2 from fitting curves >0.91.

Note on fractional saturation curves using traditional methods

In general, the dissociation constant for polymer/protein complexes relies on obtaining fluorescence quenching curves of FPs upon titration of polymer.[5–7] The quenching curves are transformed to fractional saturation curves using equation 1 and fitting with equation 2. This

simplified method is not the best model for instances in which FP is complexed but does not have a significant quench in fluorescence intensity. Using a centrifugation method provides a better insight into the interaction between the PN and FP.

Table S2. Summary of dissociation constants (K_d) for PN/protein complexes

	Dissociation Constant K_d (nM)		
	BSA	**IgG**	**R-PE**
Ph-CG	276 ± 51	352 ± 52	219 ± 42
Bn-CG	417 ± 44	733 ± 81	234 ± 37
Ph-CG'	695 ± 104	312 ± 55	341 ± 51
Bn-CG'	801 ± 106	454 ± 71	143 ± 27

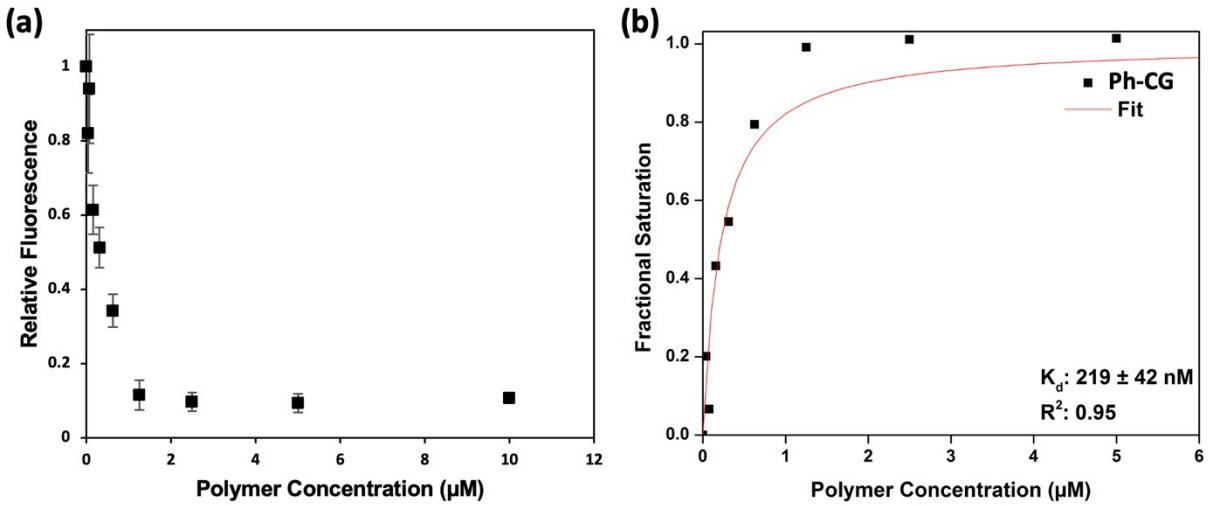


Figure S3. Representative example of relative fluorescence of free R-PE (a) and fractional saturation (b) at various concentrations of Ph-CG. Data shown represents the mean of three independent experiments ± standard deviation.

2.6 Complexation Ratio

The loading ratio of various FPs (FITC-BSA, FITC-IgG, and R-PE) was determined by measuring the fluorescence of non-complexed FPs in centrifuged complexes. PN stock solutions were prepared by dissolving dried PN powders in DMSO and diluting to final concentrations of 1 mM in DMSO. 1 mM stock solutions were serially diluted in DMSO to a final concentration of 3.91 μM. Adequate volumes of each PN stock solution was diluted in water (20% v/v) and mixed with equal volumes of FP solution for a final complex volume of 50 μL. Complexes were incubated for 30 min at room temperature before a ten-fold dilution in PBS to a final volume of 500 μL (final concentration of BSA, IgG, and R-PE was 0.5 μg/mL, 5.0 μg/mL and 0.5 μg/mL, respectively). The final concentration of PNs ranged from 10 μM

to 0 μM. The solutions were centrifuged at 22,000 x g for 10 min. The supernatant was collected in a cuvette and the fluorescence intensity of FPs in the supernatant was measured at their emission λ_{max}. The amount of FP in supernatant was calculated according to the standard curve of FP in the same solvent composition.

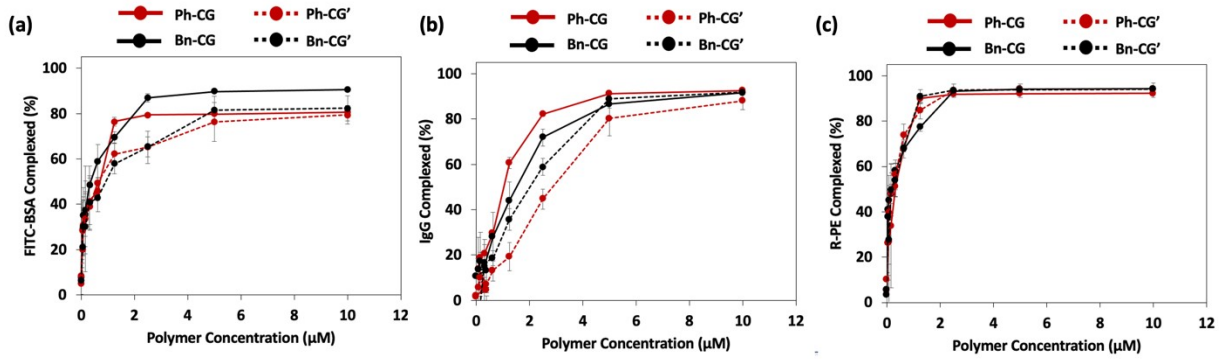


Figure S4. Complexation ratio of FITC-BSA (a), FITC-IgG (b), and R-PE (c) at various concentrations of PNs. Data shown represents the mean of three independent experiments ± standard deviation.

2.7 Serum Stability Assay

The stability of complexes was studied by monitoring the fluorescence of the R-PE released from complexes. PN stock solutions were prepared by dissolving dried PN powders in DMSO and diluting to final concentrations of 1 mM in DMSO. Adequate volumes of PN stock solutions were diluted in water (20% v/v) and mixed with equal volumes of R-PE solution for a final complex volume of 50 μL. Complexes were incubated for 30 min at room temperature before a ten-fold dilution in PBS with 10% FBS to a final volume of 500 μL (final PN/R-PE concentration was 5 μM and 2 nM, respectively). The solutions were incubated at room temperature for the respective amounts of time before being centrifuged at 22,000 x g for 10 min. The supernatant was collected in a cuvette and the fluorescence intensity of R-PE in the supernatant was measured at 574 nm. The amount of R-PE in supernatant was calculated according to the standard curve of R-PE in the same solvent composition.

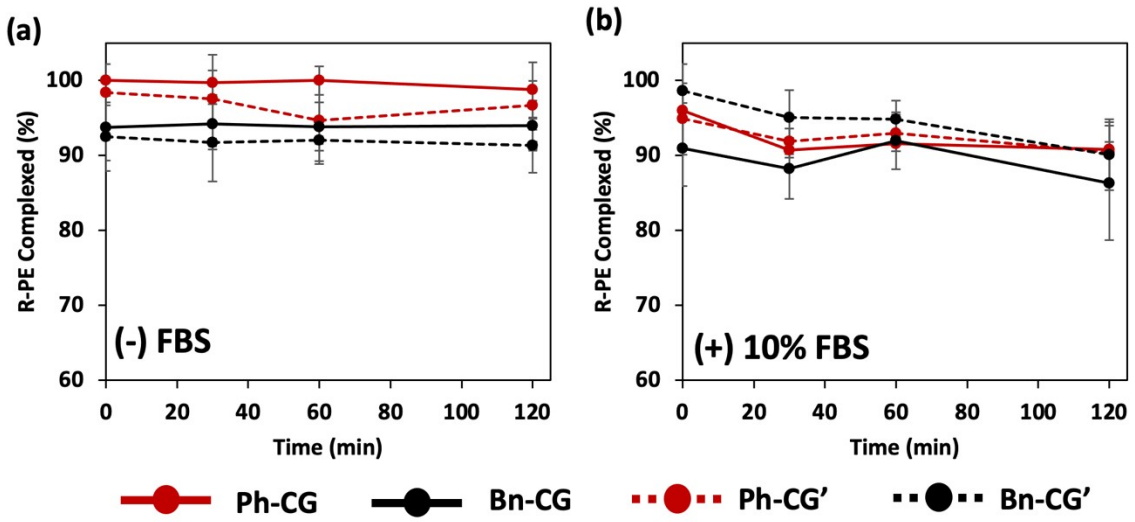


Figure S5. Stability of PN/R-PE complexes in PBS (a) and PBS containing 10% FBS (b). Data represents the mean of three independent experiments +/- standard deviation.

3. Cell Culture

HeLa, bEnd.3, and Caco-2 cells were cultured in Gibco DMEM High Glucose medium supplemented with 10% (v/v) FBS and 1% (v/v) Penicillin-Streptomycin mixture.

3.1. MTT Assay

MTT (methylthiazole tetrazolium) assay was performed with PN/protein complexes are described previously. HeLa cells were seeded in a 96-well plate (~10,000/well) in 200 µL of complete medium and allowed to attach for one day at 37 °C under a humidified atmosphere of 5% CO_2 prior to sample treatment. Serial dilutions of PNs were prepared. PNs were added to HeLa cells (final concentration of 10, 5, 2.5, 1.25 µM) and incubated for 24 h prior to MTT treatment. 10 µL of MTT solution (5 mg/mL in PBS) was added and incubated for 4 h at 37 °C. After incubation, 200 µL of medium was gently removed and 100 µL of biological grade DMSO was added to solubilize the purple formazan crystals. Absorbance was measured using a microwell plate reader. Cell viability was determined as a function of the absorbance of each sample relative to control wells. All measurements represent the average of three measurements ± standard deviation.

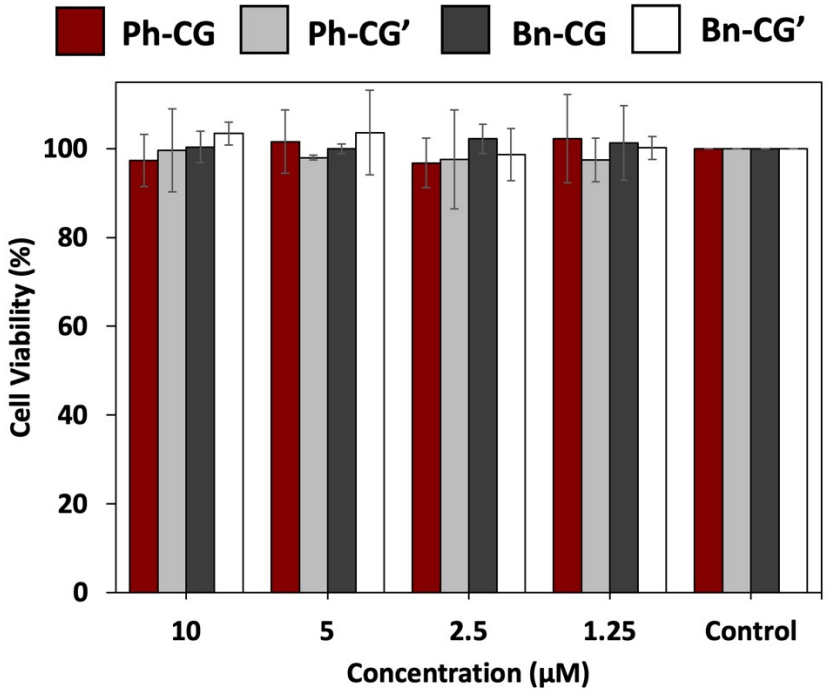


Figure S6. Cell viability assay of PNs. Data represents mean of three independent experiments ± standard deviation.

4. Protein Delivery Experiments

4.1. Flow Cytometry Analysis

Hela, bEnd.3, and Caco-2 cells were seeded into 12 well plates (~100,000/well) in complete media and allowed to attach for one day at 37 °C under a humidified atmosphere of 5% CO_2 prior to sample treatment. R-PE stock solution was diluted to working concentration with 1X PBS. Polymer stock solutions were prepared at 1 mM in DMSO. 40 μL of polymer/protein complexes were prepared by mixing appropriate volumes of polymer and protein sub-stock solutions and incubating for 30 min at room temperature in the dark. Complexes were added dropwise to each well to the cells in complete media and incubated for varying periods of time, depending on the experiment. After the incubation periods required, adherent cells were rinsed three times with full volumes of PBS, followed by washing with 1 μM heparan sulfate solution to remove any extracellular surface-bound complexes.[8,9] The cells were harvested with TrypLE, transferred to centrifuge tubes, rinsed an additional three times with PBS and once with a 0.1% Trypan Blue solution before being finally resuspended in 300 μL of PBS. Cells were analyzed by flow cytometry, in which data for 10,000 events were collected.

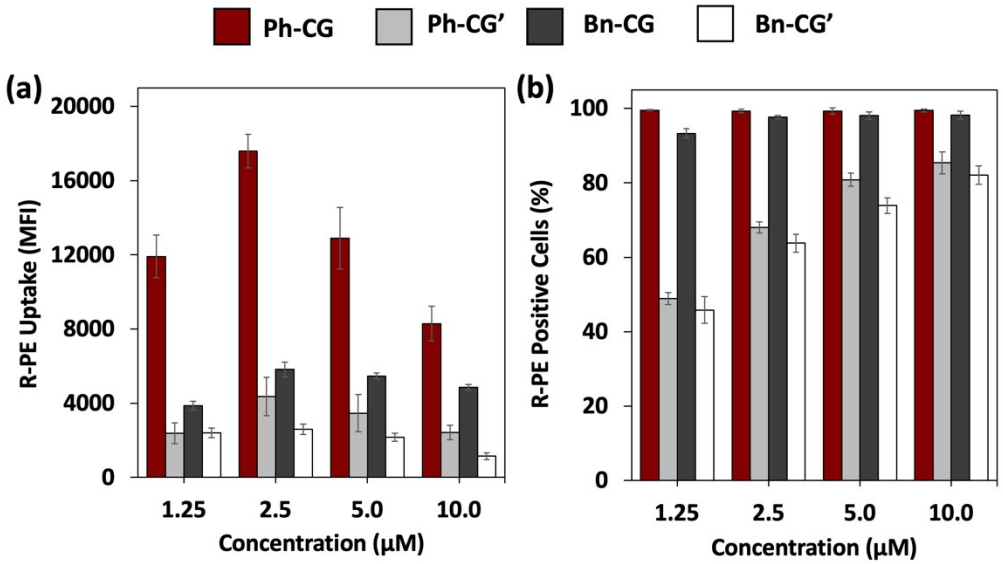


Figure S7. Median fluorescence intensity (a) and percent R-PE positive (b) of HeLa cells treated with PN/R-PE complexes for 18 h at various concentration of PNs. The concentration of R-PE was fixed at 0.5 μg/mL. Extracellular fluorescence was quenched with the addition of TB. Data shown is the mean of three independent experiments ± standard deviation.

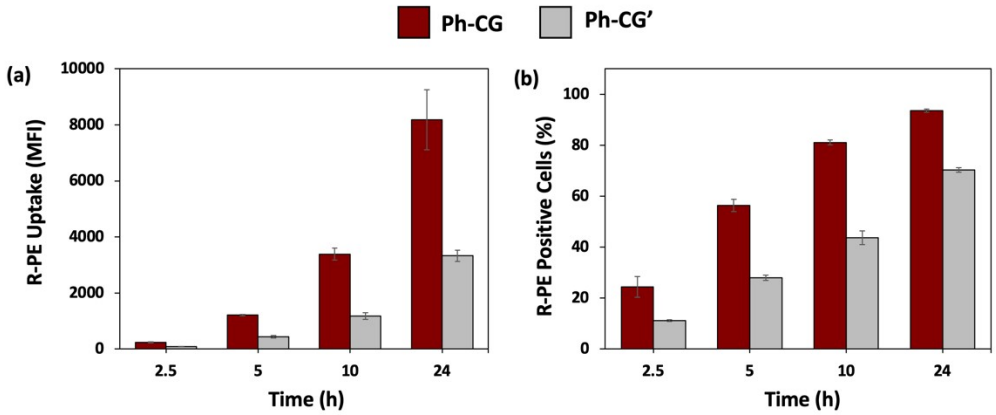


Figure S8. Median fluorescence intensity (a) and percent R-PE positive (b) of HeLa cells treated with PN/R-PE complexes for various time frames. The concentration of PNs and R-PE were 5.0 μM and 0.5 μg/mL, respectively. Extracellular fluorescence was quenched with the addition of TB. Data shown is the mean of three independent experiments ± standard deviation.

4.1.1. Delivery of PN/R-PE Complexes to Endothelial and Epithelium Cell Lines

In order to study the internalization of PN/R-PE complexes on different cell lines, flow cytometry was done using bEnd.3 and Caco-2 cells.

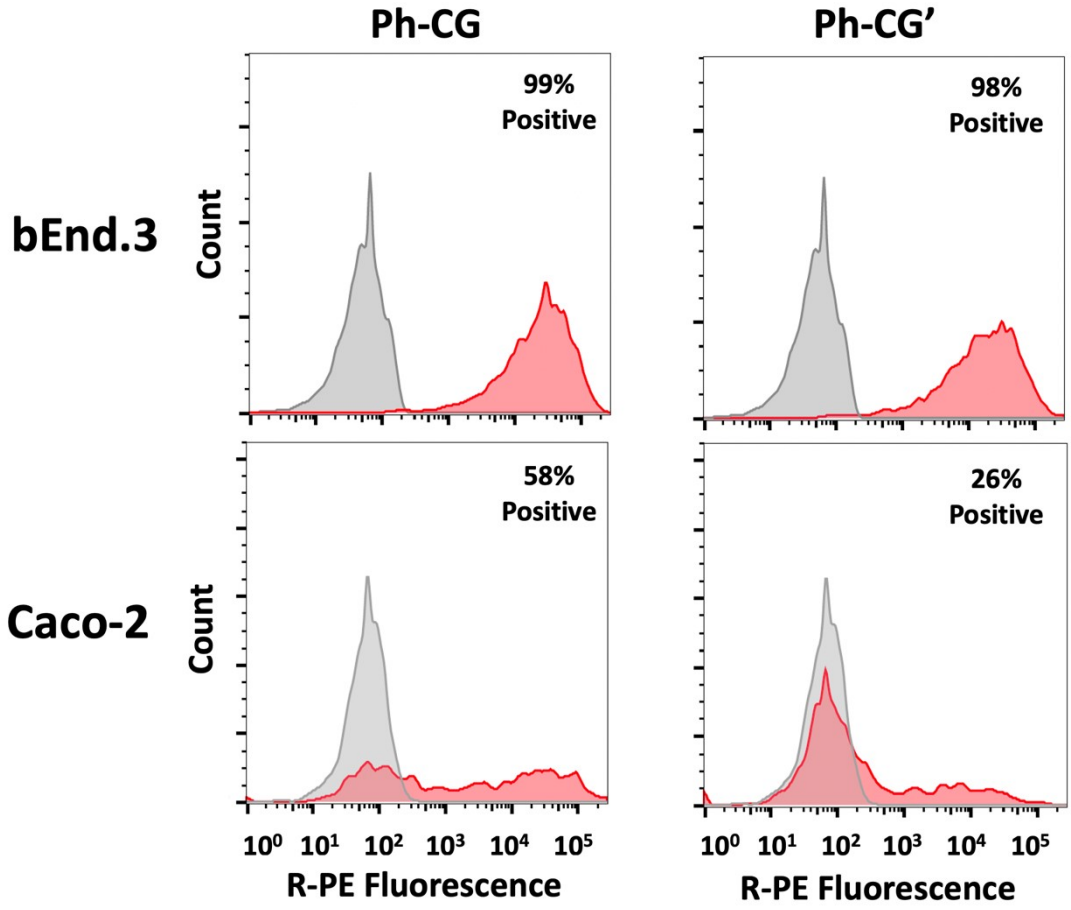


Figure S9. Flow cytometry histograms of Ph-CG and Ph-CG' mediated delivery of R-PE to bEnd.3 and Caco-2 cells after overnight incubation. The concentration of polymer and R-PE were 5.0 μM and 0.5 μg/mL, respectively.

4.1.2. Internalization of PN/R-PE Complexes

In order to study the internalization or adsorption of PN/R-PE on the cellular membrane, flow cytometry analysis was done with or without the addition of trypan blue.

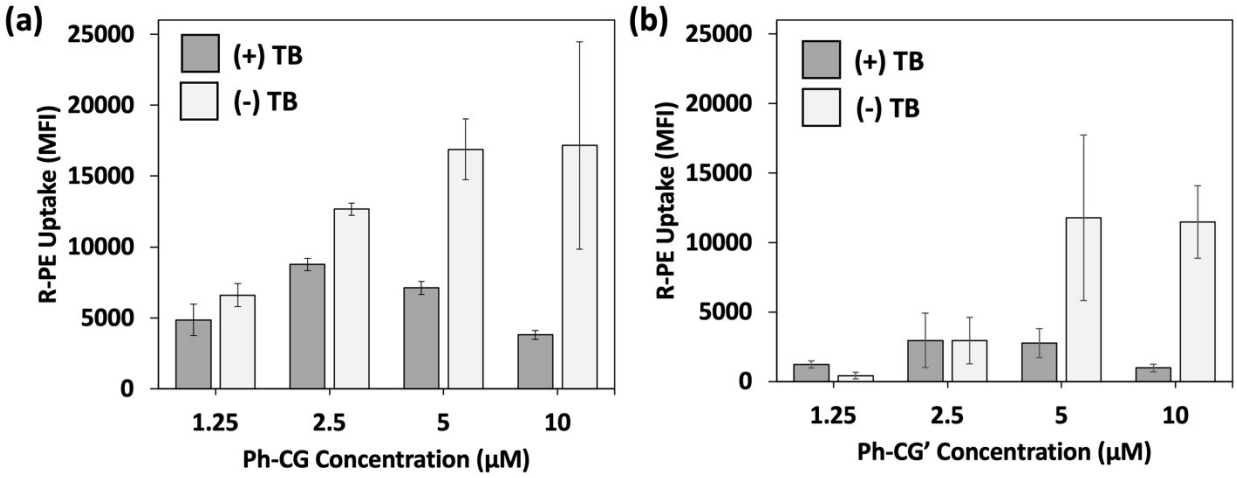


Figure S10. Median fluorescence intensity of HeLa cells treated with PN/R-PE complexes for 18 h with or without the addition of TB. The concentration of R-PE was fixed at 0.5 μg/mL. Data shown is the mean of three independent experiments ± standard deviation.

5. Cellular Entry Pathway

In order to study the mechanism of uptake, HeLa cells were treated with Ph-CG and Ph-CG'/R-PE complexes (10 μM/2 nM) for 1 h under energy-independent conditions or under pre-treatment with various pharmacological inhibitors. Briefly, HeLa cells were seeded the day prior to sample treatment in 12-well plates (100,000/well). The day of experiment, cells were equilibrated for 30 minutes under 4 °C, ATP depletion conditions (NaN_3: 10 mM & 2-deoxyglucose: 50 mM), chlorpromazine (28 μM), LYS 294003 (3 μM), Cytochalasin D (10 μM), methyl-B-cyclodextrin (1 mM), wortmannin (500 nM), EIPA (100 μM) and genistein (200 μM) or normal culture conditions. Complexes were added dropwise, and cells were incubated for 1 h prior to analysis via flow cytometry as described previously.[5]

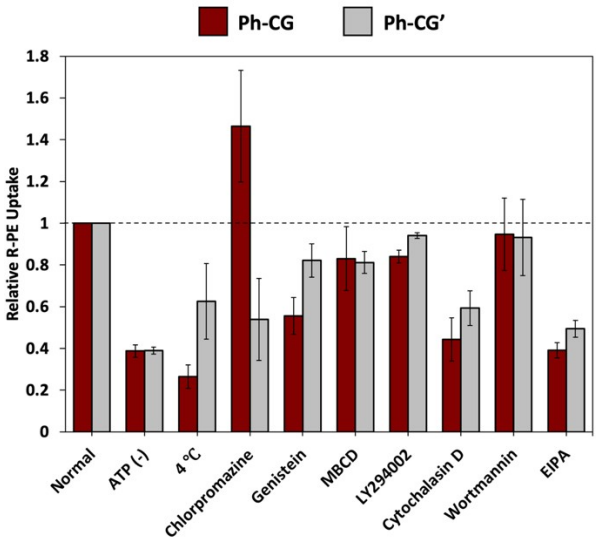


Figure S11. Relative median fluorescence intensity of HeLa cells in energy-independent conditions (ATP depletion and 4 °C) or pre-treated with various pharmacological endocytosis inhibitors followed by incubation with Ph-CG and Ph-CG'/R-PE complex for 1 h. The concentration of polymer and R-PE were 20 μM and 16 nM, respectively. Data shown is the mean of three independent experiments +/- standard deviation.

6. Confocal Imaging

Hela cells were seeded on 12-well plates (~60,000/well) containing glass coverslips one day before sample treatment. Complexes were prepared as described previously. After incubation for varying periods of time using the same culture conditions discussed earlier, the medium was removed, and cells were washed three times with PBS and once with heparan sulfate. Cells were fixed with 4% PFA for 10 minutes and rinsed once with PBS. Nuclei were stained with Hoechst 33342 at a final concentration of 1 μg/mL for 7 minutes. For cells with Lysotracker Red and ActinRed staining, the manufacturers' protocol was followed. The coverslips were mounted on microscope slides using 1:1 glycerol/PBS mounting medium.

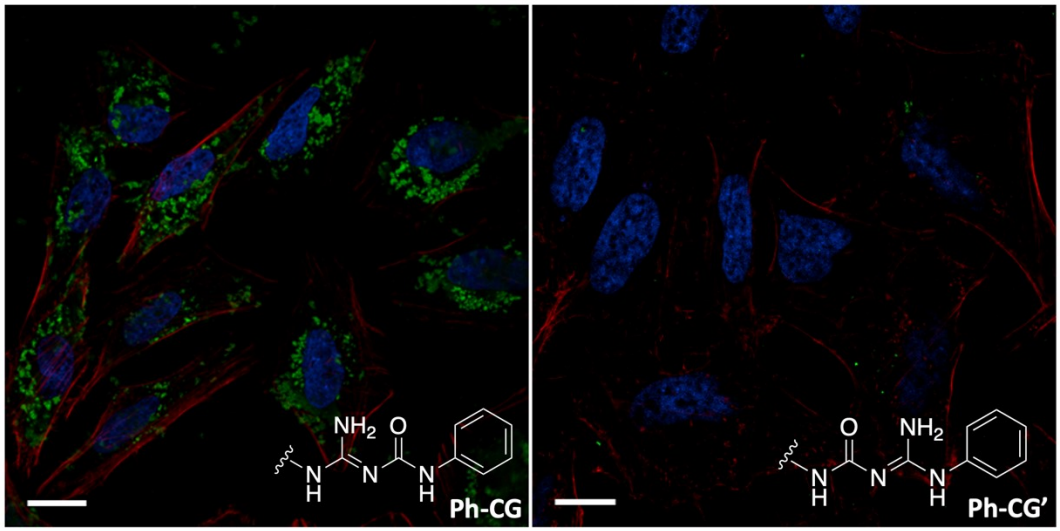


Figure S12. Confocal microscope images of HeLa cells incubated with Ph-CG and Ph-CG'/R-PE. Concentrations of PN and FITC-BSA were 10 μM and 40 nM, respectively. The mebrane was stained with ActinRed.

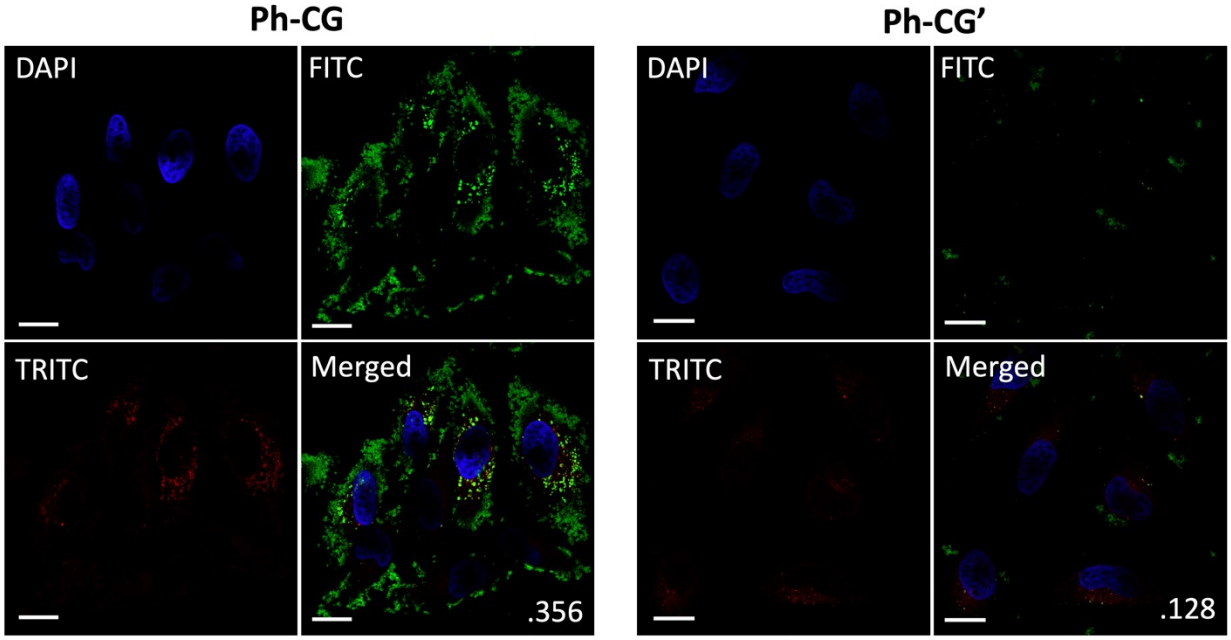


Figure S13. Confocal microscope images of HeLa cells incubated with Ph-CG and Ph-CG'/R-PE. Concentrations of PN and FITC-BSA were 10 μM and 40 nM, respectively. Lysotracker Red was used according to manufacturer guidelines. PCC values are given on the bottom right corner.

7. Functional Protein Delivery

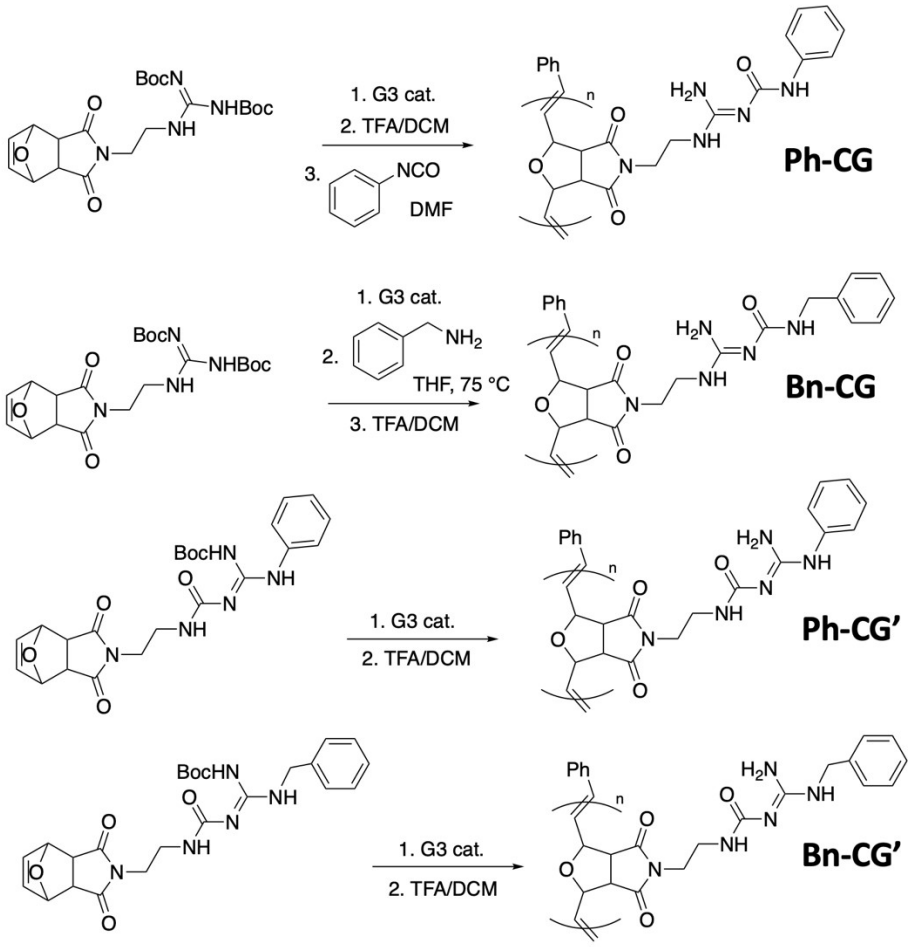
7.1. Anti-pAkt Delivery

MTT (methylthiazole tetrazolium) assay was performed with PN/protein complexes are described previously.[5] HeLa cells were seeded in a 96-well plate (~10,000/well) in 200 μL of complete medium and allowed to attach for one day at 37 °C under a humidified atmosphere of 5% CO_2 prior to sample treatment. Serial dilutions of anti-pAkt were prepared and complexed with various PNs. Complexes or anti-pAkt alone were added to HeLa cells (final concentration of anti-pAkt of 20, 10, 5, 2.5, 1.25, 0.625, and 0 μg/mL) and incubated for 48 h prior to MTT treatment. 10 μL of MTT solution (5 mg/mL in PBS) was added and incubated for 4 h at 37 °C. After incubation, 200 μL of medium was gently removed and 100 μL of biological grade DMSO was added to solubilize the purple formazan crystals. Absorbance was measured using a microwell plate reader. Cell viability was determined as a function of the absorbance of each sample relative to control wells. All measurements represent the average of three measurements ± standard deviation.

7.2. Intracellular β-Gal Delivery

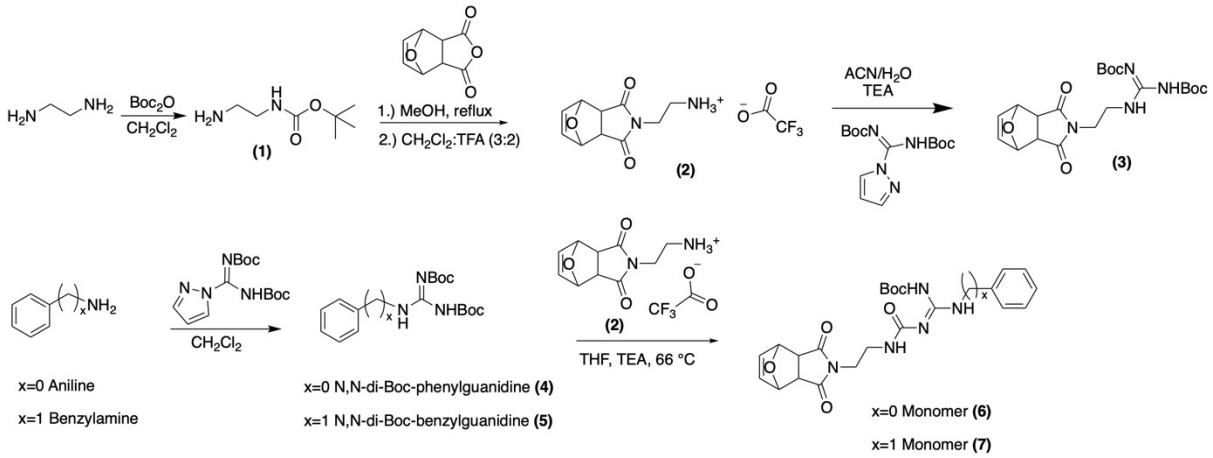
Intracellular β-Gal activity was visualized with X-Gal staining. HeLa cells were seeded in a 12-well plate (~40,000/well) in 800 μL of complete medium and allowed to attach for one day at 37 °C under a humidified atmosphere of 5% CO_2 prior to sample treatment. Dilutions of β-Galactosidase were prepared and complexed with Ph-CG for 30 min prior to addition to cells. Cells were incubated with complexes (2.5 μM and 0.5 μg/mL of PN and β-Gal, respectively) or β-Gal alone (0.5 μg/mL) overnight. After incubation, the medium was removed and cells were thoroughly rinsed with PBS (3x) and heparin Sulfate (1x, 1 mg/mL) and fixed with 4% PFA for 15 min. The X-Gal staining solution was prepared by dissolving X-Gal in DMSO (10 mg/mL) and making a 5% solution in PBS. 500 μL of the X-Gal staining solution was added to the fixed cells and incubated overnight at 37 °C in the absence of CO_2. The wells were rinsed thoroughly with PBS and imaged.

8. Synthesis



Scheme S1. General synthesis of carbamoylated polymers (Ph-CG, Bn-CG, Ph-CG', Bn-CG').

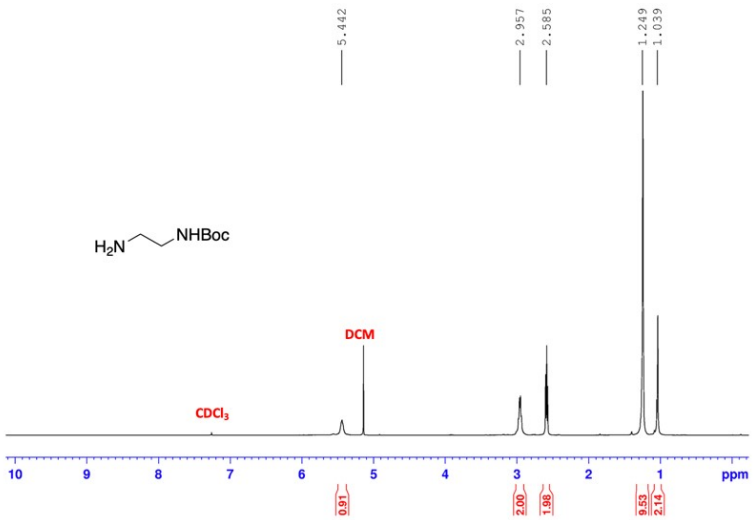
8.1. Monomer Synthesis



Scheme S3. Synthesis of monomers used for polymerization.

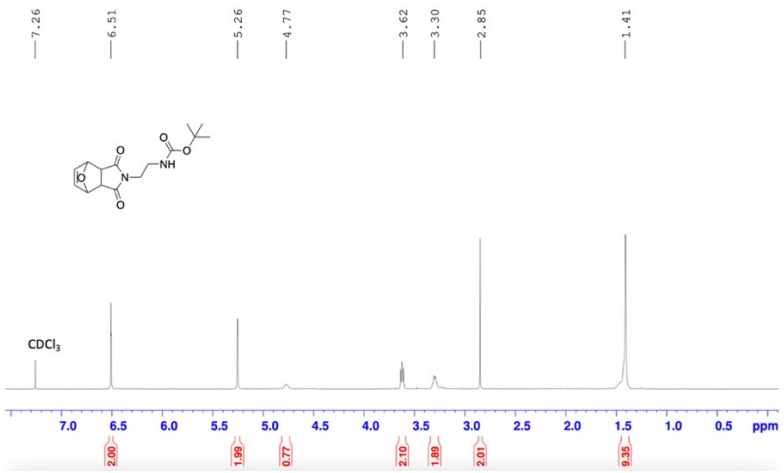
Synthesis of 1. To a round bottom flask containing a solution of ethylenediamine (1.0 mol, 66.6 mL) in 800 mL of methylene chloride, di-*tert*-butyl dicarbonate anhydrous (0.15 mol, 32.7 g) pre-mixed in 300 mL of methylene chloride was added dropwise over 2 hours. The reaction continued for 16 hours; then, the organic phase was washed with H_2O (2x 800 mL), brine (1x 800 mL), dried ($MgSO_4$ anhydrous), and filtered. The solvent was removed in vacuo, and a light-yellow viscous liquid was obtained.

1: Yield: 78%. ^{1}H NMR (400 MHz, $CDCl_3$): δ 5.44 (br s, 1H), 2.96 (s, 2H), 2.59 (t, 2H), 1.25 (s, 9H), 1.04 (s, 2H).

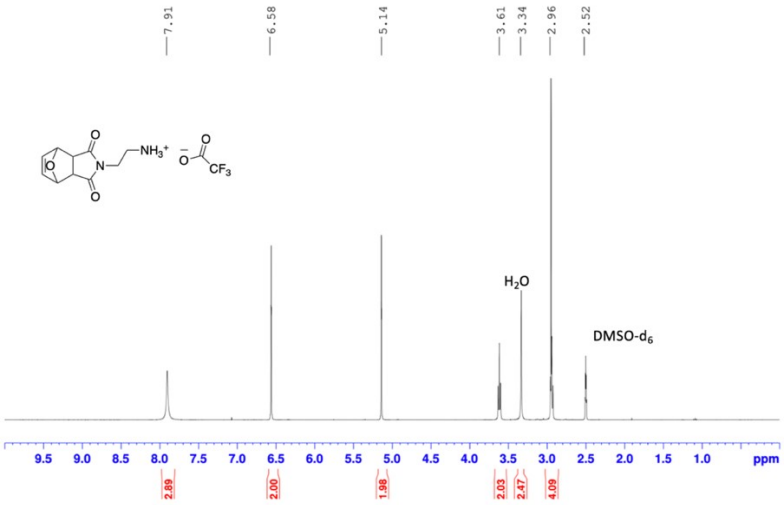


Synthesis of 2. *N*-Boc-ethylenediamine (39.2 mmol, 6.35 g) and exo-7-oxabicyclo [2.2.1] hept-5-ene-2,3-dicarboxylic anhydride (30.1 mmol, 5 g) were dissolved in 100 mL of MeOH. Then, triethylamine (72 mmol, 10 mL) was added, and the reaction mixture was stirred in reflux. The heating stopped after 20 hours, and the compound precipitated once the crude reached room temperature. The product was purified by recrystallization using methylene chloride and methanol mixture. The solid obtained was deprotected using methylene chloride: trifluoracetic acid mixture (3:2). The solvent was removed in vacuo, and the crude was purified by precipitation in ether solution yielding pure compound 2.

2 (protected): Yield: 62%. ^{1}H NMR (400MHz, $CDCl_3$): δ 6.51 (s, 2H), 5.26 (s, 2H), 4.77 (br s, 1H), 3.62 (t, 2H), 3.30 (br q, 2H), 2.85 (s, 2H), 1.41 (br s, 9H).

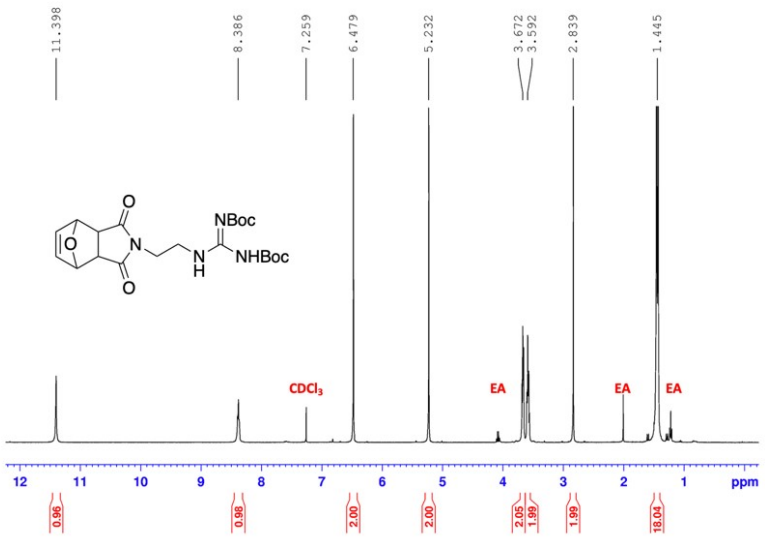


2: Yield: 59%. ^{1}H NMR (400MHz, DMSO-d_6): δ 7.91 (s, 3H), 6.58 (s, 2H), 5.14 (s, 2H), 3.61 (t, 2H), 2.96 (br s, 4H).



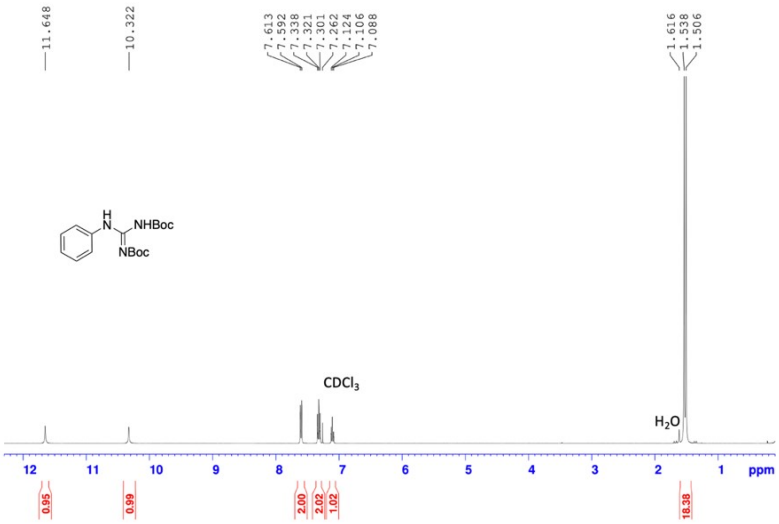
Synthesis of 3. Compound 2 was deprotected using TFA and the free amine was converted to Boc-protected guanidine using *N,N′*-Di-Boc-1*H*-pyrazole-1-carboxamidine.

3: Yield: 68%. ^{1}H NMR (400 MHz, $CDCl_3$): δ 11.40 (s, 1H), 8.39 (s, 1H), 6.48 (s, 2H), 5.23 (s, 2H), 3.67 (t, 2H), 3.60 (t, 2H), 2.84 (s, 2H), 1.44 (m, 18H).



Synthesis of 4. *N, N′*-di-Boc-1*H*-pyrazole-1-carboxamidine (3.22 mmol, 1 g) was added to a round bottom flask and dissolved in methylene chloride. Aniline (4.2 mmol, 0.38 mL) was added to the flask, and the reaction mixture was heated at 50 °C and stirred for 16 hours. The crude was diluted to 80 ml of methylene chloride and washed with H_2O (2x100 mL), brine (1x100 mL), dried ($MgSO_4$ anhydrous), and filtered. The solvent was removed in vacuo, and the crude was purified by recrystallization using hot MeOH, compound 4.

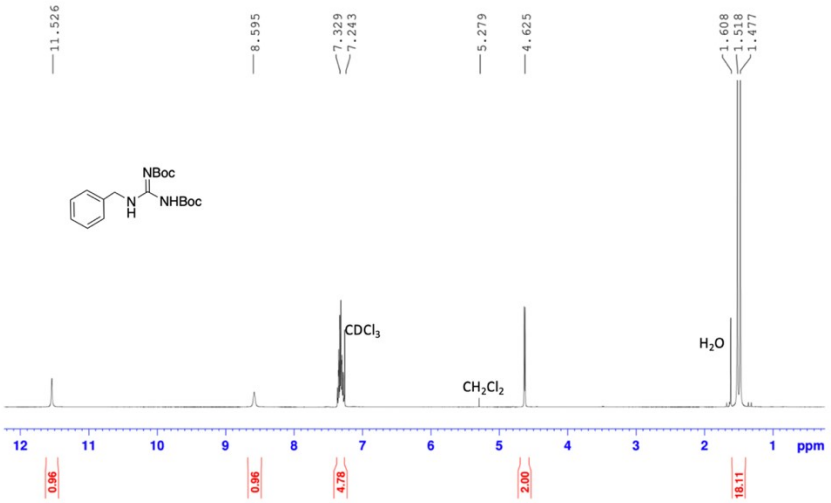
4: ^{1}H NMR (400MHz, $CDCl_3$): δ 11.65 (s, 1H), 10.32 (s, 1H), 7.60 (d, 2H), 7.32 (t, 2H), 7.11 (t, 1H), 1.54 and 1.51 (br s, 18H).



Synthesis of 5. *N, N′*-di-Boc-1*H*-pyrazole-1-carboxamidine (11 mmol, 3.4 g) was added to a round bottom flask and dissolved in methylene chloride. Triethylamine (9.3 mmol, 1.3 mL) and Benzylamine (9.3 mmol, 1.02 mL) were added to the flask, and the reaction mixture was stirred for 16 hours at room temperature. The crude was diluted to 80 ml of methylene chloride and

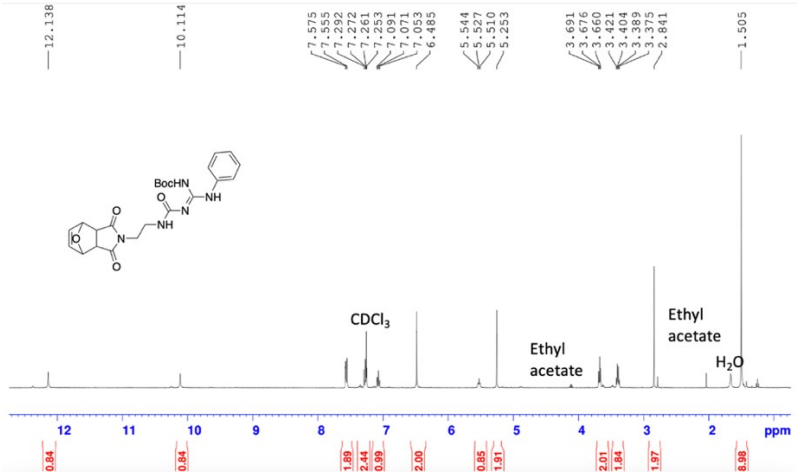
washed with H_2O (2x100 mL), brine (1x100 mL), dried ($MgSO_4$ anhydrous), and filtered. The solvent was removed in vacuo, and the crude was purified by recrystallization using methylene chloride:MeOH, yielding compound 5.

5: Yield: 63%. ^{1}H NMR (400MHz, $CDCl_3$): δ 11.53 (s, 1H), 8.60 (s, 1H), 7.33 (br m, 5H), 4.63 (s, 2H), 1.52 and 1.48 (br s, 18H).



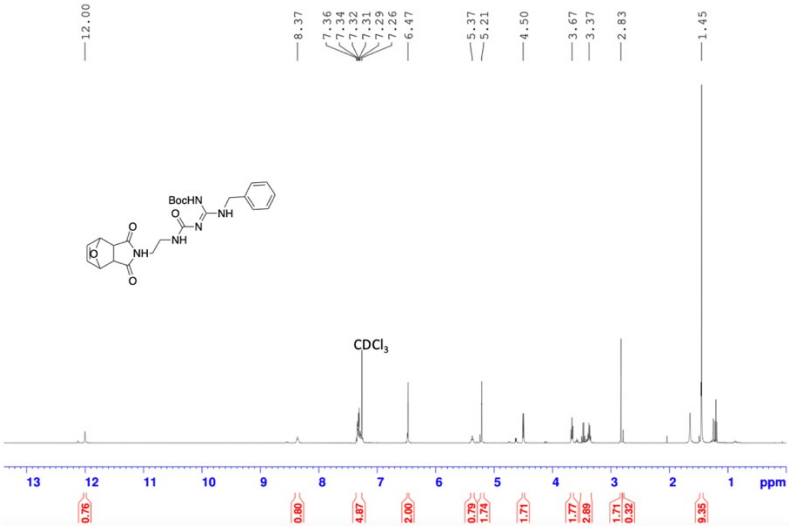
Synthesis of 6. Compound 2 (1.24 mmol, 400 mg) and *N, N*-di-Boc-phenylguanidine **4** (0.992 mmol, 333 mg) were added to a round bottom flask and dissolved in 6 mL of THF and triethylamine (7.44 mmol, 1 mL). The mixture was stirred at reflux for 16 hours. Then, the solvent was removed in vacuo, and the crude was diluted in methylene chloride and washed with NH_4Cl (1x10 mL), brine (1x10 mL), dried ($MgSO_4$ anhydrous), and concentrated in vacuo. The crude was purified by flash column using n-Hexane: Ethyl Acetate (9:1) and then (4:1), yielding monomer 6.

6: Yield: 52%. ^{1}H NMR (400MHz, $CDCl_3$): δ 12.14 (s, 1H), 10.11 (s, 1H), 7.56 (d, 2H), 7.27 (t, 2H), 7.07 (t, 1H), 6.49 (s, 2H), 5.53 (t, 1H), 5.25 (s, 2H), 3.69 (t, 2H), 3.40 (m, 2H), 2.84 (s, 2H), 1.50 (s, 9H).



Synthesis of 7. Compound 2 (1.24 mmol, 400 mg) and *N, N*-di-Boc-benzylguanidine 5 (0.992 mmol, 346.6 mg) were added to a round bottom flask and dissolved in 6 mL of THF and triethylamine (7.44 mmol, 1 mL). The mixture was stirred at reflux for 16 hours. Then, the solvent was removed in vacuo, and the crude was diluted in methylene chloride and washed with NH_4Cl (1x10 mL), brine (1x10 mL), dried ($MgSO_4$ anhydrous), and concentrated in vacuo. The crude was purified by flash column using n-Hexane: Ethyl Acetate (2x 4:1) and (1x 1:1) yielding monomer 7.

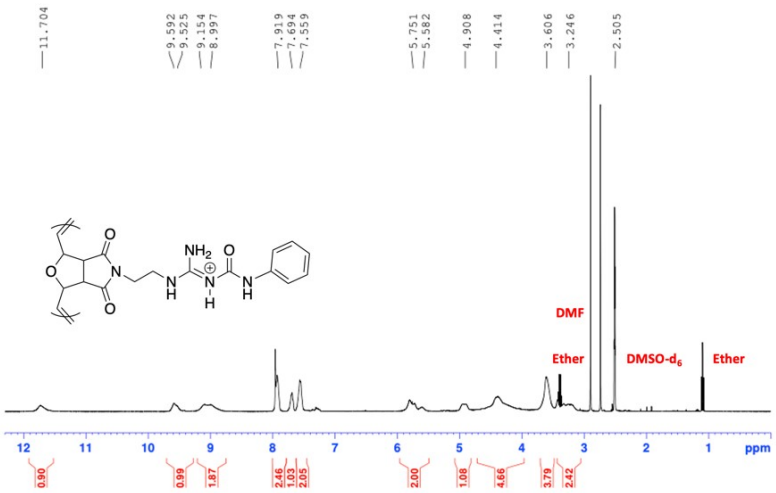
7: Yield: 45%. ^{1}H NMR (400MHz, $CDCl_3$): δ 12.00 (s, 1H), 8.37 (s, 1H), 7.32 (br m, 5H), 6.47 (s, 2H), 5.37 (t, 1H), 5.21 (s, 2H), 4.50 (s, 2H), 3.67 (t, 2H), 3.37 (m, 2H), 2.83 (s, 2H), 1.45 (s, 9H).



8.2. Polymer Synthesis

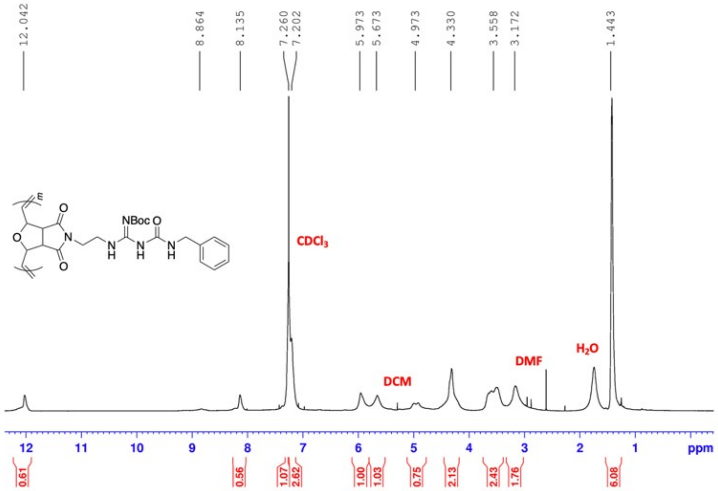
Synthesis of Ph-CG. Monomer 3 was dissolved in DCM. Grubbs 3rd generation catalyst was dissolved in DCM and added to the stirring monomer solution. After 60 min, the living polymer was end-capped with 1 mL of ethyl vinyl ether. The polymer solution was precipitated into stirring diethyl ether (3x) and dried. The dried solid was dissolved in a DCM/TFA mixture (1:1, v/v) and deprotected overnight. The reaction mixture was precipitated (3x) and collected via centrifugation. Deprotected polymer was dissolved in dry DMF. 5 equivalents of phenyl isocyanate were added. The mixture was sealed in a vial and allowed to react overnight at 75 °C. The reaction mixture was precipitated into diethyl ether (3x) and the polymer was collected via centrifugation.

Ph-CG: ^{1}H NMR (400 MHz, DMSO-d_6): δ 11.70 (br, 1H), 9.59 (br, 1H), 9.15 (br, 2H), 7.91 (br, 2H), 7.69 (br, 1H), 7.55 (br, 2H), 5.75 (br, 2H), 4.91 (br, 1H), 4.41 (br, 1H), 3.60 (br, 4H), 3.24 (br, 2H).

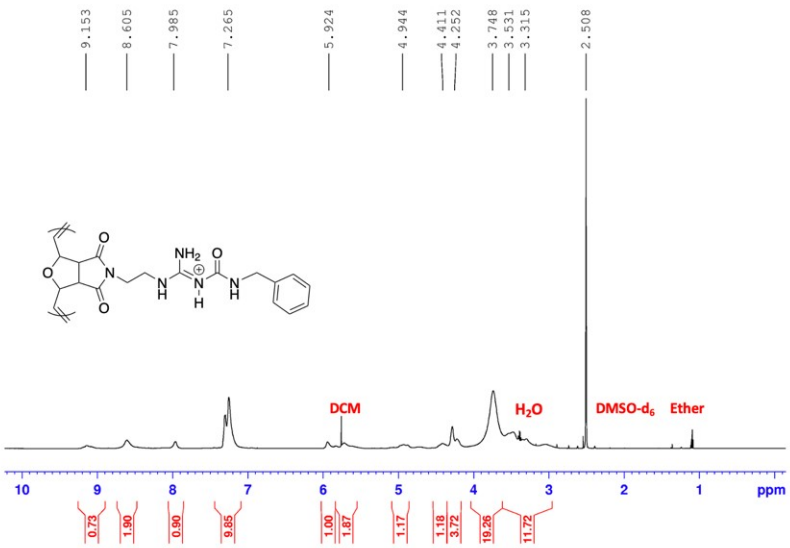


Synthesis of Bn-CG. Monomer 3 was dissolved in DCM. Grubbs 3rd generation catalyst was dissolved in DCM and added to the stirring monomer solution. After 60 min, the living polymer was end-capped with 1 mL of ethyl vinyl ether. The polymer solution was precipitated into stirring diethyl ether (3x) and dried. The dried polymer was dissolved in THF to which 2.5 equivalents of benzylamine were added. The mixture was sealed in a vial and allowed to react overnight at 75 °C. The reaction mixture was precipitated into diethyl ether (3x) and then deprotected in DCM/TFA mixture (1:1, v/v). Deprotected polymer solution was precipitated into diethyl ether (3x) and polymer was collected via centrifugation.

Bn-CG protected: ^{1}H NMR (400 MHz, $CDCl_3$): δ 12.04 (br, 1H), 8.14 (br, 1H), 7.20 (br, 5H), 5.97 (br, 1H), 5.67 (br, 1H), 4.97 (br, 1H), 4.33 (br, 3H), 3.56 (br, 4H), 3.17 (br, 2H), 1.44 (s, 9H).

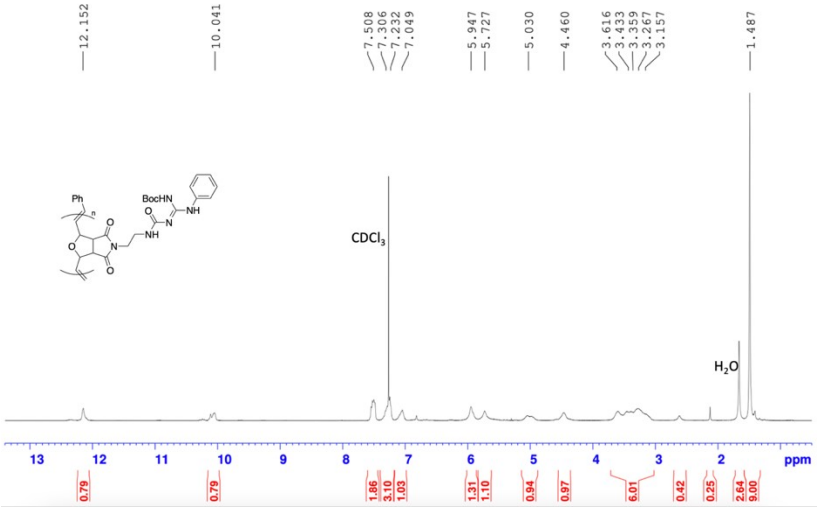


Bn-CG: ^{1}H NMR (400 MHz, DMSO-d_6): δ 9.15 (br, 1H), 8.61 (br, 2H), 7.99 (br, 1H), 7.27 (br, 5H), 5.92 (br, 1H), 5.71 (br, 2H), 4.94 (br, 1H), 4.41 (br, 1H), 4.25 (br, 4H), 3.75 (br, 4H).

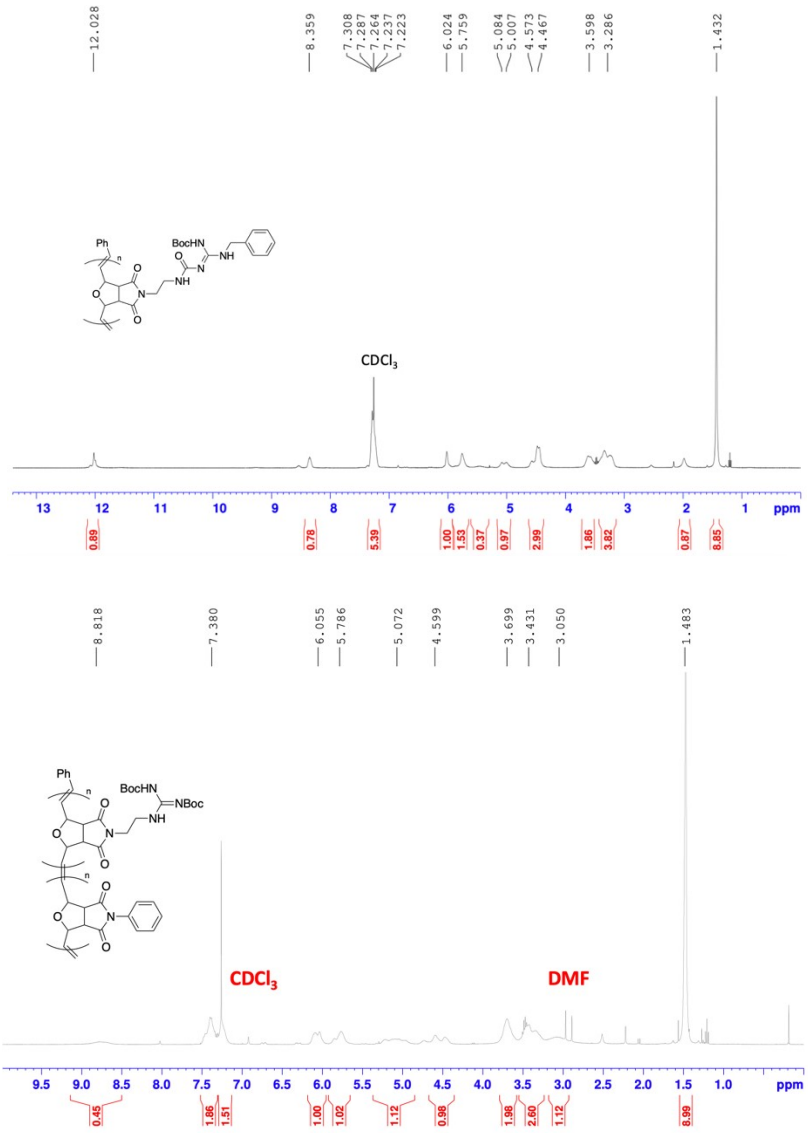


Synthesis of Ph-CG' and Bn-CG'. Grubb's catalyst 3rd generation was dissolved in dry DCM and added to a stirring solution containing monomer 6 (Ph-CG') or 7 (Bn-CG'). After 60 min, the living polymer was end-capped with 1 mL of ethyl vinyl ether. The polymer solution was precipitated into stirring diethyl ether (3x) and dried. The protected polymer Ph-CG' was deprotected by mixing it with methylene chloride and trifluoroacetic acid (3:2) for 4 hours. Then, the deprotected polymer was precipitated in diethyl ether (3x) and dried.

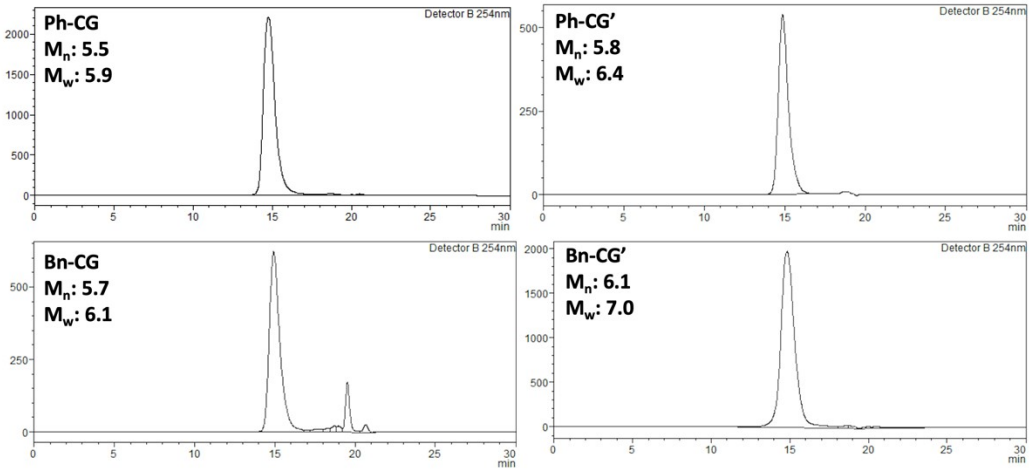
Ph-CG' (protected): ^{1}H NMR (400MHz, $CDCl_3$): δ 12.15 (br s, 1H), 10.04 (br m, 1H), 7.50 (br m, 2H), 7.30 (br m, 3H), 7.05 (br s, 1H), 5.95 (br s, 1H), 5.73 (br s, 1H), 5.03 (br m, 1H), 4.46 (br s, 1H), 3.36 (br m, 6H), 1.49 (br s, 9H).



Bn-CG' (protected): ^{1}H NMR (400MHz, $CDCl_3$): δ 12.03 (br m, 1H), 8.36 (br s, 1H), 7.26 (br m, 5H), 6.02 (br s, 1H), 5.76 (br s, 1H), 5.08 (br m, 1H), 4.50 (br m, 3H), 3.60 (br m, 2H), 3.29 (br m, 4H), 1.43 (br s, 9H).



GPC chromatogram of PNs



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