Electronic Supplementary Material (ESI) for ChemComm.

Supporting Information

Amphipathic poly-β-peptides for intracellular protein delivery†

Qianyi Ren,[a‡] Qi Chen,[b‡] Lanfang Ren,[a] Chuntao Cao,[b] Runhui Liu,*[b] Yiyun Cheng*[a]

[a] Shanghai Frontiers Science Center of Genome Editing and Cell Therapy, Shanghai Key Laboratory of Regulatory Biology, School of Life Sciences, East China Normal University, Shanghai, 200241, P. R. China

[b] State Key Laboratory of Bioreactor Engineering, Key Laboratory for Ultrafine Materials of Ministry of Education, Frontiers Science Center for Materiobiology and Dynamic Chemistry, Research Center for Biomedical Materials of Ministry of Education, School of Materials Science and Engineering, East China University of Science and Technology, Shanghai, 200237, China

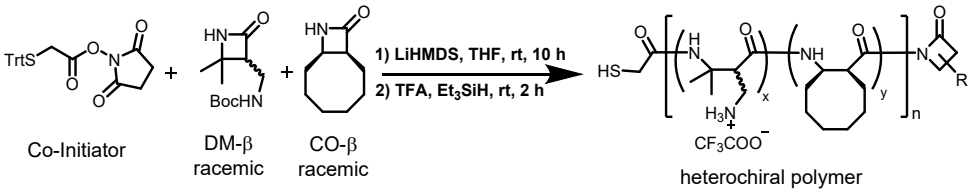
‡ The authors contributed equally to the work.

Table of Contents

1. Materials

The chemical reagents and solvents used in the synthesis of Pbps were purchased from Adamas-beta® without further purification. PULSin™ was obtained from Polyplus-transfection (France). Genistein (GEN), chlorpromazine (CPZ) and saporin were purchased from Sigma-Aldrich (USA). Isopropyl-b-$_D$-thiogalactopyranoside (IPTG), kanamycin, methyl-β-cyclodextrin (MβCD) were purchased from Macklin (Shanghai, China). Ethylisopropylamiloride (EIPA) and bafilomycin A1 (Baf A1) were purchased from MedChemExpress (MCE, Shanghai, China). β-Galactosidase (β-Gal) and ribonuclease A (RNase A) were obtained from J&K Scientific (Shanghai, China). Enhanced Cell Counting Kit 8 and Annexin V-FITC/PI Apoptosis Kit were purchased from Elabscience (Wuhan, China). BCA Protein Assay Kit, hoechst 33342, LDH assay kit and In Situ β-galactosidase Staining Kit were from Beyotime (Jiangsu, China). Lysotracker red was from Life technologies (USA). Fluorescein di-β-galactopyranoside (FDG) was purchased from Cayman (USA). Ni-NTA Sefinose (TM) Resin 6FF (Settled Resin) was purchased from Sangon (Shanghai, China). Dulbecco's modified Eagle's medium, fetal bovine serum (FBS), trypsin, penicillin and streptomycin were purchased from Gibco (Thermo Fisher).

2. Synthesis of poly-β-peptides



R represents either the side chain of DM or CO subunit

Poly-β-peptide Pbp-4 was synthesized as a heterochiral polymer with an average chain length of 35 amino acid residues, by following the protocol in precedent literature[1]. Firstly, DM-β[2] (±)-3-tert-Butyloxycarbonylaminomethyl-4,4-dimethyl azetidin-2-one (28.5 mg, 0.125 mmol), CO-β[3,4] cis-9-aza-bicyclo[6.2.0]decan-10-one (19.1 mg, 0.125 mmol) and the co-initiator[5] 2-(tritylthio) acetic acid succinimidoester (2.7 mg, 0.00625 mmol) were dissolved in anhydrous tetrahydrofuran (THF). Then lithium hexamethyldisilazide (LiHMDS) (2.6 mg, 0.0156 mmol) was added to the reaction mixture. The reaction was

stirred at room temperature for 10 hours, and then 2 drops of methanol were added to terminate the polymerization. After the solvent was removed with N_2, the mixture was dissolved in THF (1 mL), and then cold petroleum ether (45 mL) was added into the tube to precipitate out the polymer. The polymer was collected by centrifugation and then was subjected to two more cycles of dissolution/precipitation. The collected polymer was dried under vacuum to give a white solid.

Deprotection of the polymer was achieved by treating the polymer in 3 mL trifluoroacetic acid supplemented with 0.15 mL triethylsilane. After shaking for 2 hours at room temperature, the resulting solution was concentrated by N_2 and dispersed in 1 mL MeOH, followed by slow addition of 45 mL cold methyl tert-butyl ether to precipitate out the polymer as a solid. The polymer was isolated by centrifugation and then was subjected to two more cycles of dissolution/precipitation. The collected polymer was dried under vacuum to give a white solid. The solid was dissolved in water and filtered with a 0.45 μm polyether sulfone membrane, followed by lyophilization to give the β-peptide polymer as a white flocculent solid.

Other poly-β-peptides were synthesized and purified in the same steps as aforementioned Pbp-4 using feeding ratios of monomers and other reagents with the amount as shown in Table 1.

Table 1 The amount of DM-β (DM), CO-β (CO), co-initiator (I), LiHMDS for poly-β-peptides' synthesis

DM-CO	Compd	Equiv.	Mmol	Amount
Pbp-1	CO	0.5	0.125	19.1 mg
	DM	0.5	0.125	28.5 mg
	I	0.2	0.05	21.6 mg
	LHMDS	0.5	0.125	20.9 mg
Pbp-2	CO	0.5	0.125	19.1 mg
	DM	0.5	0.125	28.5 mg
	I	0.1	0.025	10.8 mg
	LHMDS	0.25	0.0625	10.5 mg
Pbp-3	CO	0.5	0.125	19.1 mg
	DM	0.5	0.125	28.5 mg
	I	0.05	0.0125	5.4 mg
	LHMDS	0.125	0.03125	5.2 mg
Pbp-4	CO	0.5	0.125	19.1 mg
	DM	0.5	0.125	28.5 mg
	I	0.025	0.00625	2.7 mg

	LHMDS	0.0625	0.015625	2.6 mg
Pbp-5	CO	0.5	0.125	19.1 mg
	DM	0.5	0.125	28.5 mg
	I	0.0125	0.003125	1.4 mg
	LHMDS	0.03125	0.015625	2.6 mg
Pbp-6	CO	0.15	0.6	91.9 mg
	DM	0.1	0.4	91.3 mg
	I	0.00625	0.025	10.8 mg
	LHMDS	0.015625	0.0625	10.5 mg
Pbp-7	CO	0.1	0.4	61.3 mg
	DM	0.15	0.6	136.9 mg
	I	0.00625	0.025	10.8 mg
	LHMDS	0.015625	0.0625	10.5 mg
Pbp-8	CO	0.075	0.3	45.9 mg
	DM	0.175	0.7	159.7 mg
	I	0.00625	0.025	10.8 mg
	LHMDS	0.015625	0.0625	10.5 mg
Pbp-9	CO	0.05	0.2	30.6 mg
	DM	0.2	0.8	182.5 mg
	I	0.00625	0.025	10.8 mg
	LHMDS	0.015625	0.0625	10.5 mg
Pbp-10	CO	0.025	0.1	15.3 mg
	DM	0.225	0.9	205.3 mg
	I	0.00625	0.025	10.8 mg
	LHMDS	0.015625	0.0625	10.5 mg
Pbp-11	DM	0.25	1	228.2 mg
	I	0.00625	0.025	10.8 mg
	LHMDS	0.015625	0.0625	10.5 mg

3. Enhanced green fluorescent protein (EGFP) expression and purification

The *Escherichia coli* (*E. coli*) BL21 cells transformed with EGFP plasmid was cultured in 10-mL lysogeny broth (LB) medium containing kanamycin (50 mg/L) at 37 ^{o}C overnight. The LB medium was added into 1 L LB medium with 1 mL kanamycin (50 mg/L), shaken at 37 ^{o}C until the OD_{600} reached 0.7 and then added with 1 mL isopropyl-b-$_{D}$-thiogalactopyranoside (IPTG). The BL21 cells were cultured at 30 ^{o}C and 200 rpm for 6 h before harvest. The cells were then resuspended in NTA buffer (20 mM Tris-HCl, 150 mM NaCl, pH 8.0) and sonicated at 0 ^{o}C to get a clear lysis solution. The solution was then

centrifuged and the supernatant was filtered with a 0.22 μm filter and then added into Ni-NTA chromatography. The Ni-NTA column was eluted by 5 mL imidazole (10 mM) to remove the protein impurities and then eluted by 15 mL imidazole (300 mM) to collect the EGFP. The collected EGFP solution was dialyzed by PBS for further purification and the concentration of EGFP in the solution was measured by a BCA Protein Assay Kit according to the manufacture's protocol.

4. Cell experimental procedures

Cell culture and protein delivery experiments

143B cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco) containing 10% FBS (Gibco), penicillin (100 μg/mL) and streptomycin (100 μg/mL) in a cell incubator (5% CO_2, 37 °C). The cells were seeded in 48-well plates overnight before protein delivery experiments. Generally, EGFP (1 mg/mL in deionized water) was mixed with Pbps (1 mg/mL in deionized water) at a final concentration of 16 μg/mL (both EGFP and Pbps) for 10 min in DMEM. The water insoluble polypeptide Pbp6 was dissolved in dimethyl sulfoxide at 10 mg/mL before complexation. The prepared samples were treated with 143B cells for 4 h, and the culture media were replaced with DMEM containing 10% FBS. The cells were further cultured for up to 20 h. At scheduled intervals, the cells were collected and treated with trypan blue (0.04%wt in PBS) to quench the fluorescence from EGFP on cell surface, followed by flow cytometry analysis (FCM, BD LSR Fortessa, USA). For laser scanning confocal microscope (CLSM) analysis, the cells were cultured in confocal dishes and treated as described above before imaging (Leica SP5, Germany). The commercial protein delivery reagent PULSin was tested as the positive control according to the manufacture's protocol.

Cell viability assay by Enhanced Cell Counting Kit 8 (CCK8)

The viability of cells was evaluated by a CCK8 (Elabscience, China). Generally, 143B cells were seeded in the 96-well plates overnight, and then treated with Pbps (1.6 μg in 100 μL FBS-free DMEM) for 24 h (After 4 h incubation, the culture media were changed to DMEM containing 10% FBS). The cells were then washed with PBS before addition of CCK8 (10 μL in 100 μL DMEM) into each well, and further cultured in the cell incubator for 2 h. After that, the optical density (OD) of each well at 450 nm was measured by a

microplate reader (Multiskan GO, Thermo Scientific). The treated cells were defined as the dosing group, while untreated cells were considered as the control group. Three repeats were conducted for each sample.

The synthesized Pbps (Pbp1-11) were also tested at different concentrations (12, 16, 20, 24 μg/mL) on 143B cells by CCK8. The experimental procedure is as described above.

Endocytosis and endosomal escape of Pbp-4/EGFP complexes.

The 143B cells were pretreated with GEN (700 μM), CPZ (20 μM), MβCD (10 mM), EIPA (100 μM), Baf A1 (300 nM) for 1 h, and then washed with PBS, and treated with the Pbp-4/EGFP complexes as described above for 24 h. After treatment, the cells were analyzed by flow cytometry or CLSM to determine the internalized EGFP. The cells without inhibitor pre-treatment were tested as a control.

For endosomal escape analysis, 143B cells were treated with Pbp-4/EGFP complexes for 1, 2, 3, 4, 6, 8 h as described above, and further stained by Lysotracker red (Life technologies, USA) for 30 min and Hoechst 33342 (Beyotime, China) for 10 min before photographed by LSCM.

Intracellular delivery of saporin and RNase A

143B cells were seeded in 96-well plates overnight and then treated with Pbp-4/saporin (0, 0.01, 0.05, 0.1, 0.25, 0.5, 0.75, 1 μg/mL saporin) or Pbp-4/RNase A (0, 6.25, 12.5, 25, 50, 100, 200 μg/mL RNase A) complexes at different protein concentrations in 100 μL DMEM. The concentration of Pbp-4 was fixed at 16 μg/mL. After 4 h incubation, the culture media were changed to DMEM containing 10% FBS. The cells were further cultured for 20 h before lactate dehydrogenase (LDH) release (Beyotime, China) according to the manufacture's protocols. Three repeats were done for each sample.

For Annexin V-FITC/PI double staining assay, the concentrations of saporin and RNase A were fixed at 0.5 μg/mL and 1 μg/mL, respectively. The concentrations of Pbp-4 and PULSin were fixed at 16 μg/mL and 4 μg/mL, respectively. The transfection step is the same as the transfection step in LDH assay. The cells were tested by FCM according to the manufacture's protocols of Annexin V-FITC/PI Apoptosis Kit.

Intracellular delivery of β-Gal

143B cells were seeded into 48-well plates, and then treated with Pbp-4/β-Gal complexes. The concentrations of Pbp-4 and β-Gal were 16 and 20 μg/mL, respectively.

PULSin/β-Gal complexes were tested as a positive control according to the manufacture's protocol. The protein delivery experiments were conducted for 24 h as described above. After treatment, the cells were washed by PBS for three times, and the cells were stained by a In Situ β-galactosidase Staining Kit. Generally, each well was added with 125 μL of staining fixative for 15 min and washed by PBS for three times. Then, 125 μL of staining working solution containing 5% X-Gal was added into each well and incubated overnight at 37 ^{o}C without CO_2. The cells were then washed by PBS and photographed by a microscope (Olympus, Japan). Besides X-Gal staining, the treated cells were also stained by the fluorescein di-β-galactopyranosid (FDG) substrate. Generally, the cells were added with 250 μL of FDG solution (30 μM, diluted with DMEM) for 4 h, and then washed with PBS and photographed by a fluorescence microscope under blue light excitation.

Characterization of Pbp/EGFP complexes

Pbp-4 was mixed with EGFP at concentrations equal to those in protein delivery experiments in 500 μL DMEM for 10 min. The size and morphology of formed Pbp-4/EGFP complexes were characterized by dynamic light scattering (DLS, Malvern Zetasizer Nano ZEN3600, UK) and transmission electron microscope (TEM, HT7700, Hitachi, Japan), respectively. In addition, the fluorescence spectra of EGFP and Pbp-4/EGFP complex solutions were measured by a fluorescence spectroscopy (Hitachi F-4500, Japan).

Protein loading capability of Pbps

The Pbps/EGFP complexes were prepared as described above and diluted to one-fourth of original concentration by DMEM, and the solutions were centrifuged at 13000 rpm for 20 min at 4 ^{o}C. The EGFP concentrations in the supernatant solutions were measured by a fluorescence spectroscopy. The excitation wavelength (Ex) was 450 nm and the emission wavelength (Em) interval was from 480 to 700 nm. For EGFP standard curve, the protein stock solution was diluted by DMEM to 0.5~4 μg/mL, and the fluorescence intensity of these samples were monitored to prepare the standard curve ($F=1356*C+1720$, R^2 = 0.9909, where F is the fluorescence intensity, and C is the concentration of EGFP, μg/mL). The protein loading ratio of Pbps was calculated by (fed EGFP dose - free EGFP dose) / fed EGFP dose.

5. ^{1}H NMR spectra of poly-β-peptides

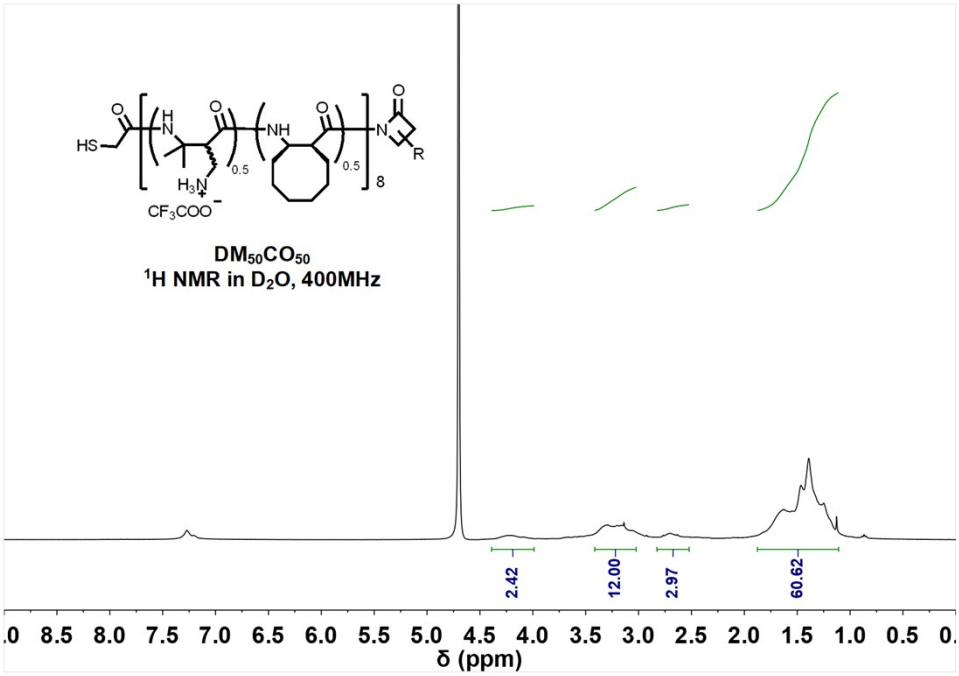


Figure S1. ^{1}H NMR spectrum of Pbp-1 in D_2O.

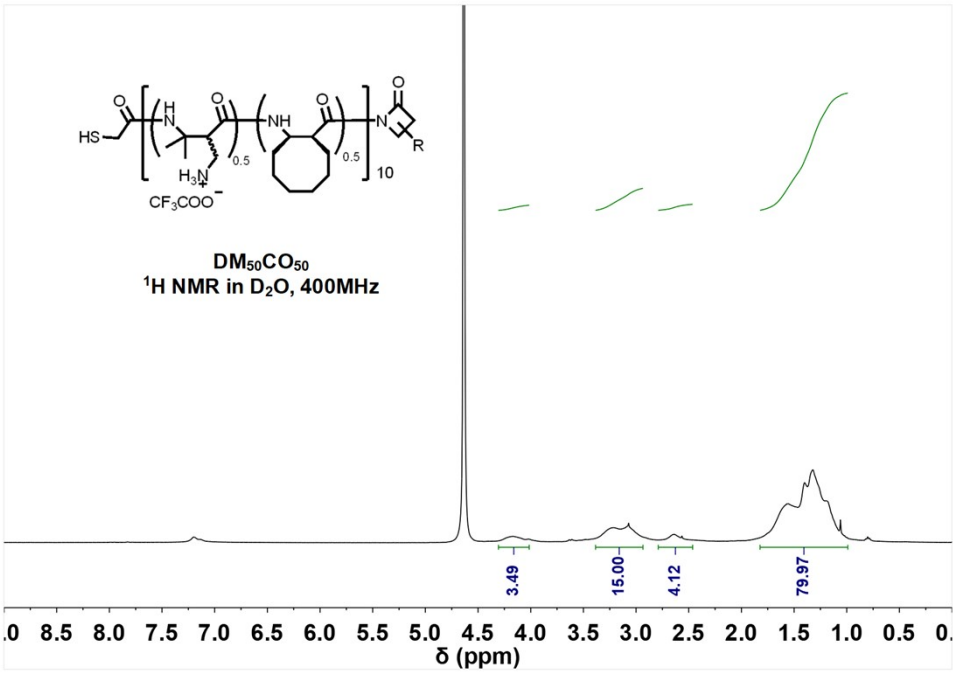


Figure S2. ^{1}H NMR spectrum of Pbp-2 in D_2O.

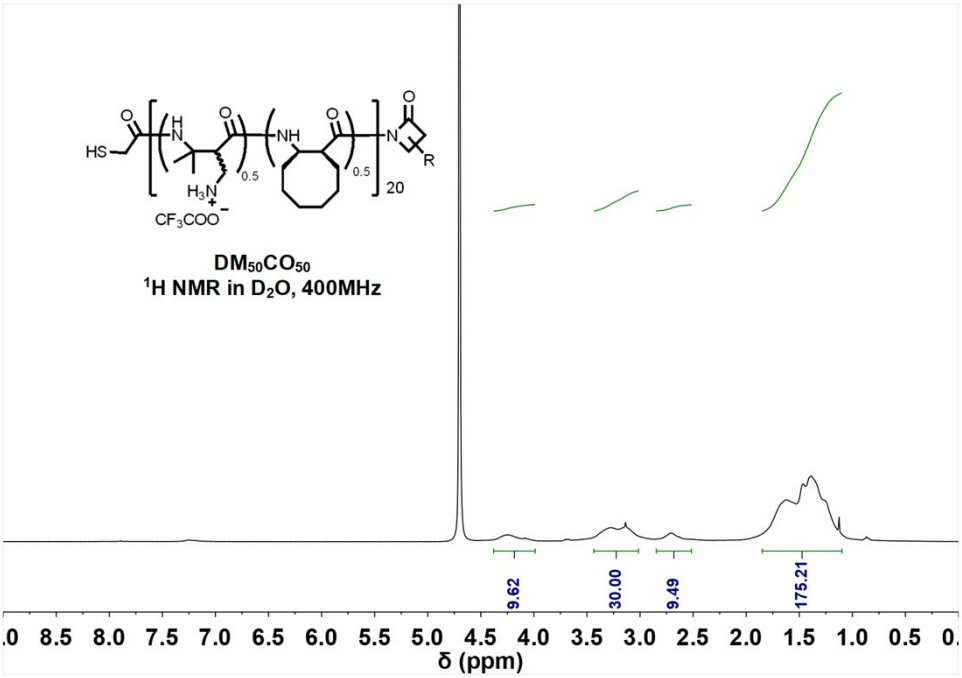


Figure S3. ^{1}H NMR spectrum of Pbp-3 in D_2O.

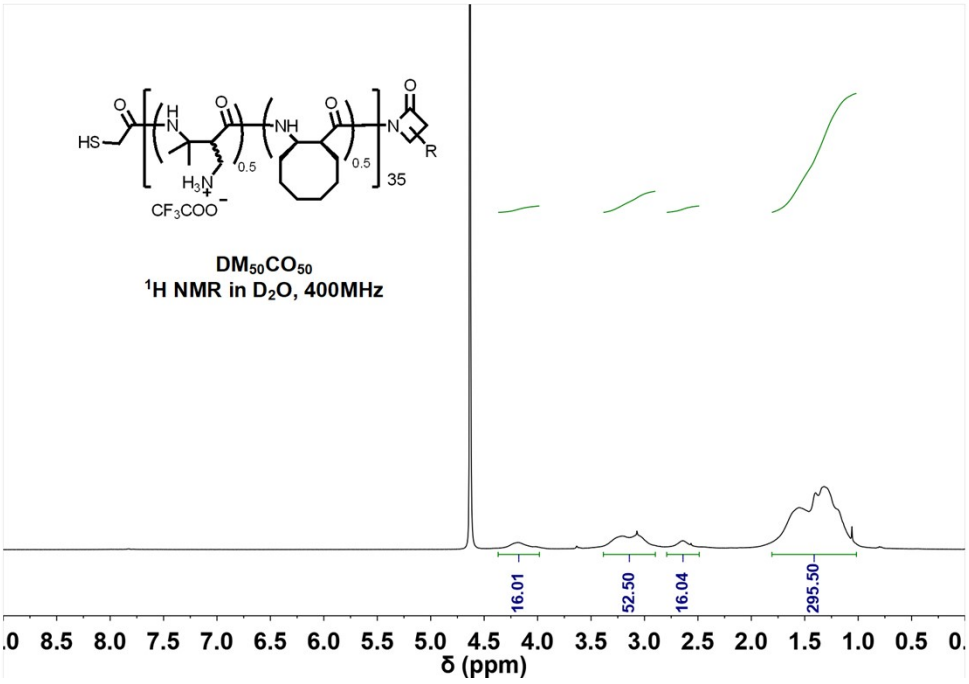


Figure S4. ^{1}H NMR spectrum of Pbp-4 in D_2O.

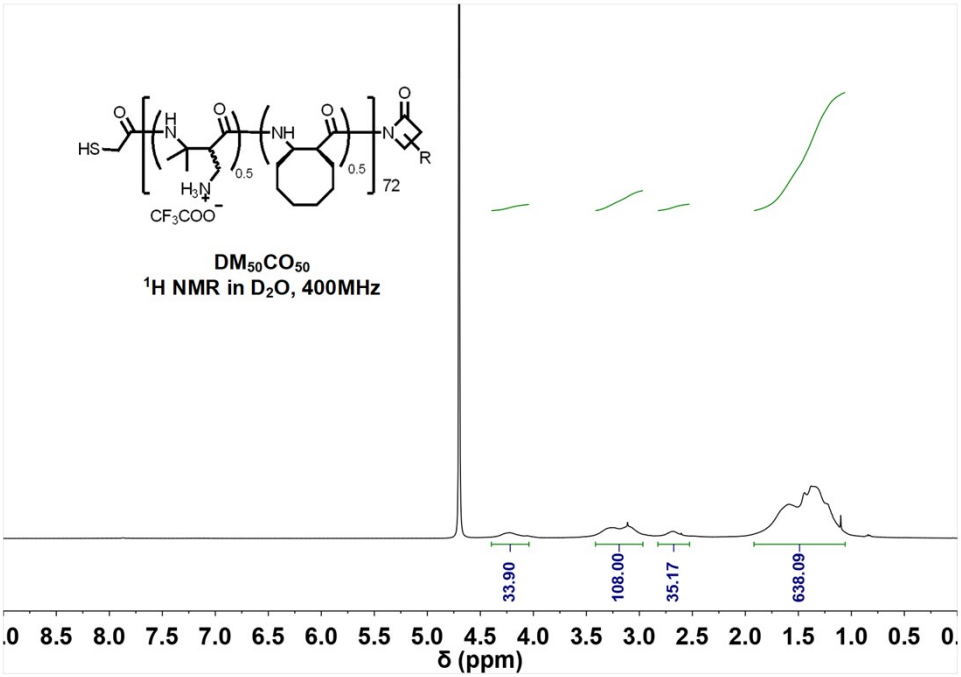


Figure S5. ^{1}H NMR spectrum of Pbp-5 in D_2O.

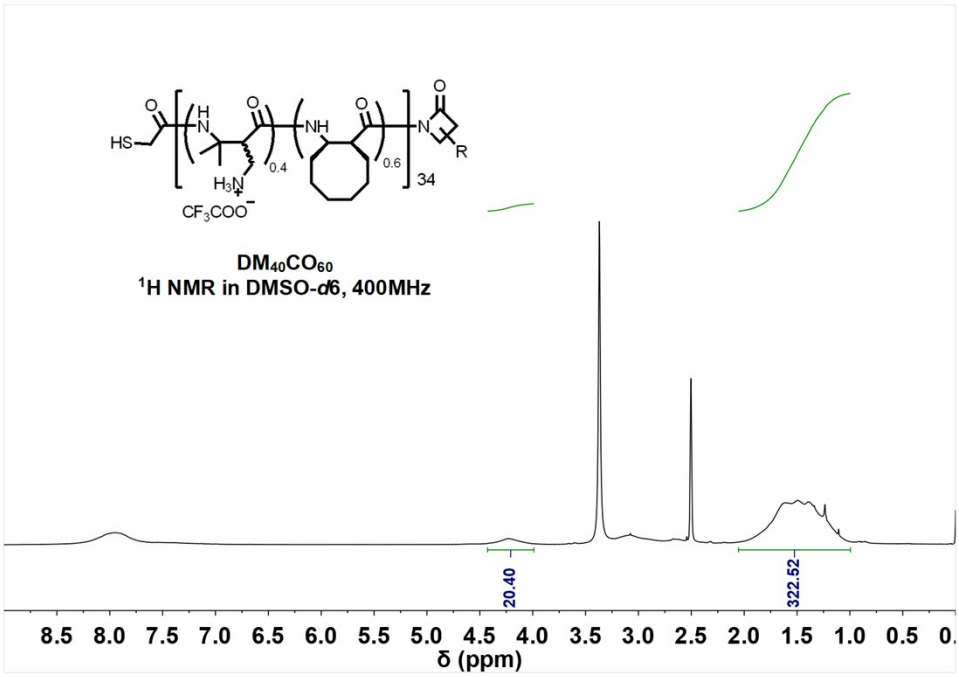


Figure S6. ^{1}H NMR spectrum of Pbp-6 in DMSO-d6.

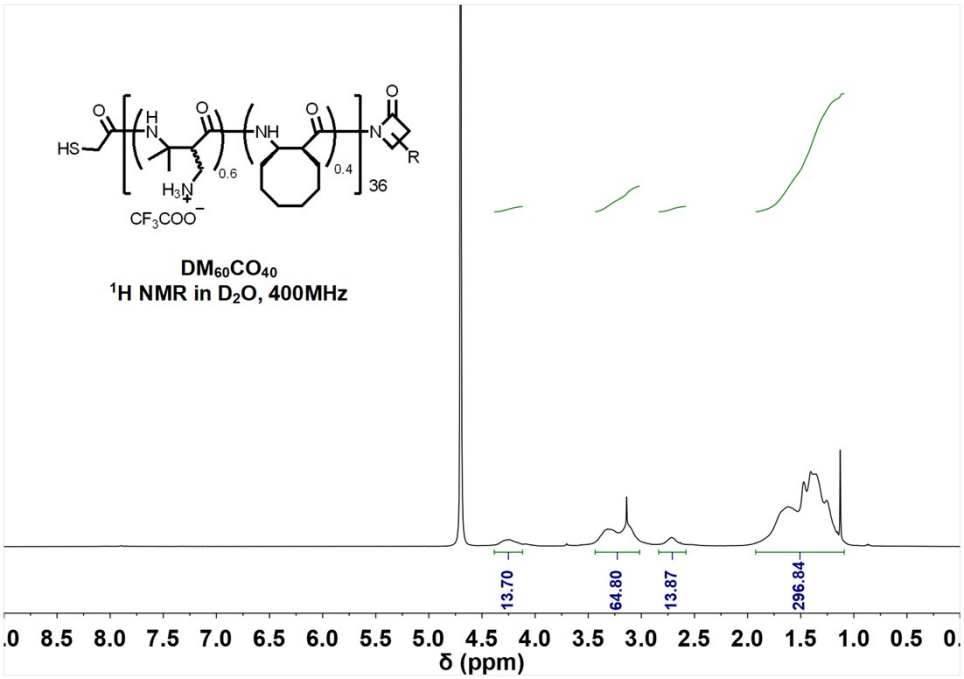


Figure S7. ^{1}H NMR spectrum of Pbp-7 in D_2O.

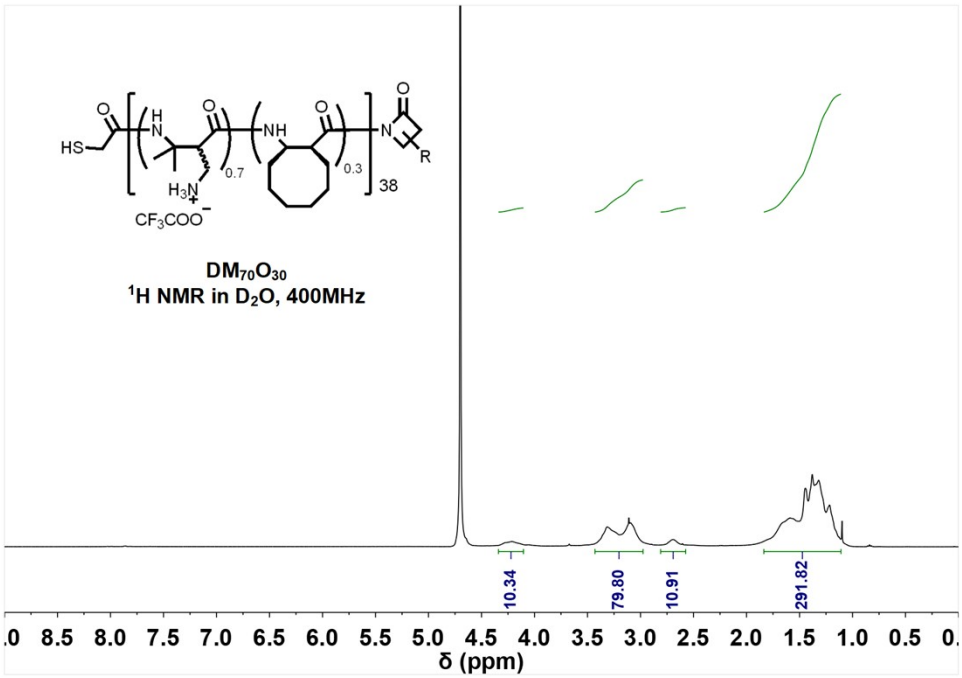


Figure S8. ^{1}H NMR spectrum of Pbp-8 in D_2O.

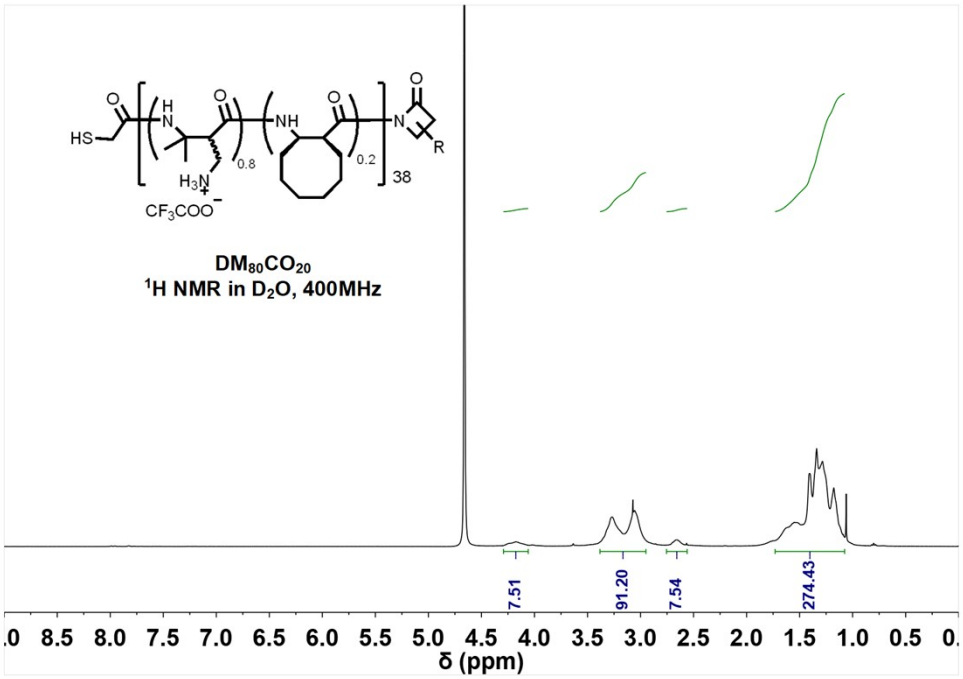


Figure S9. ^{1}H NMR spectrum of Pbp-9 in D_2O.

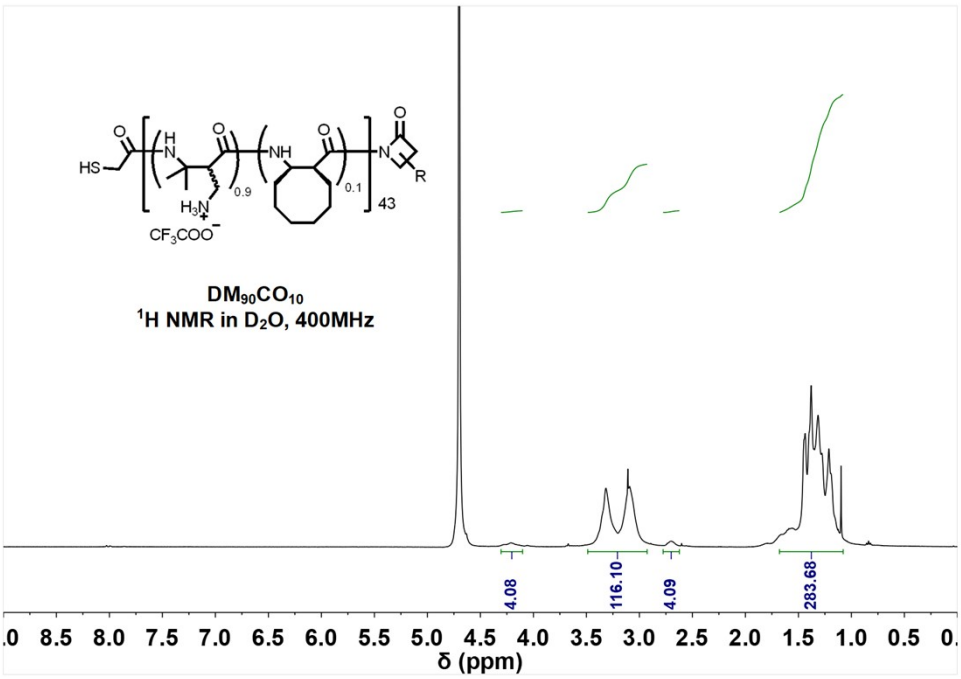


Figure S10. ^{1}H NMR spectrum of Pbp-10 in D_2O.

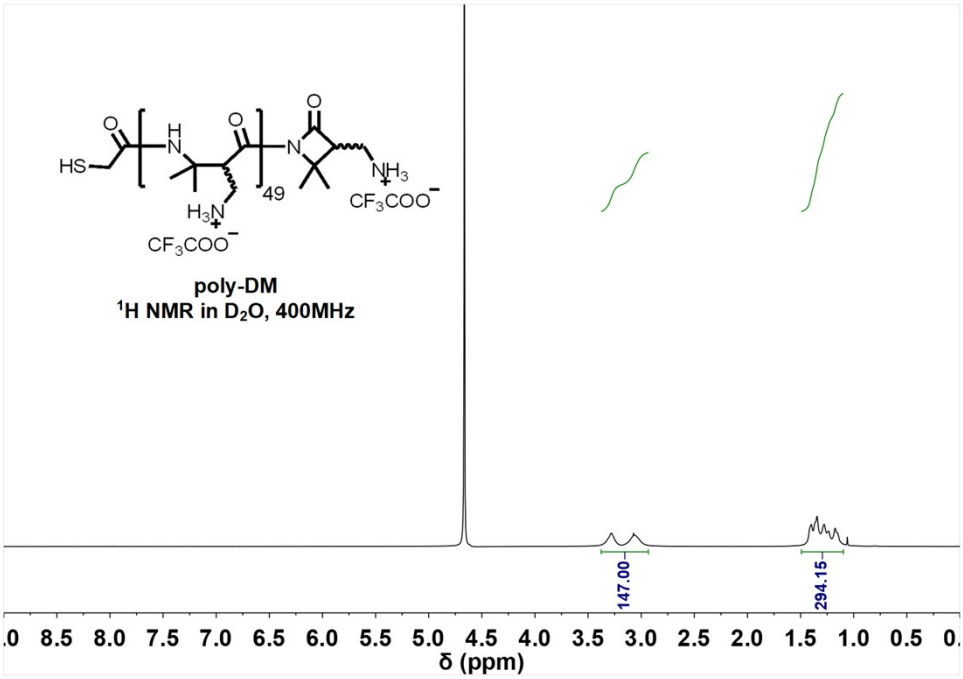


Figure S11. ^{1}H NMR spectrum of Pbp-11 in D_2O.

6. Other cell experiments results

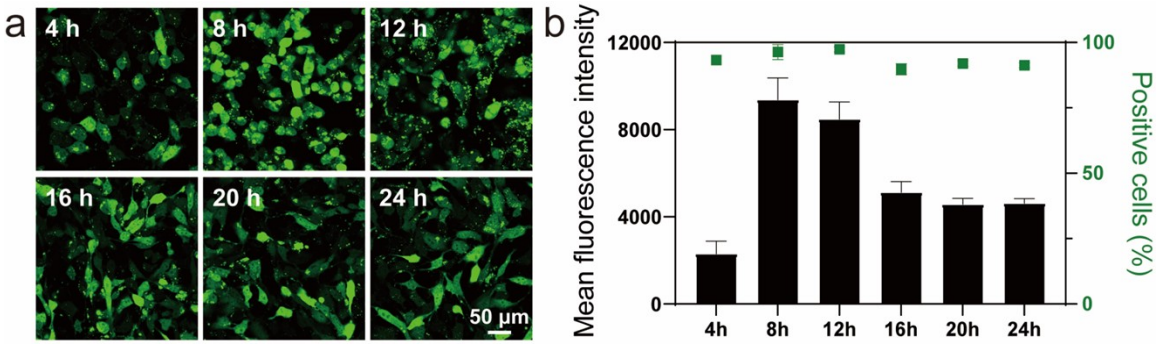


Figure S12. Confocal images (a) and mean fluorescence intensity (b) of 143B cells incubated with Pbp-4/EGFP complexes for 4, 8, 12, 16, 20, 24 h.

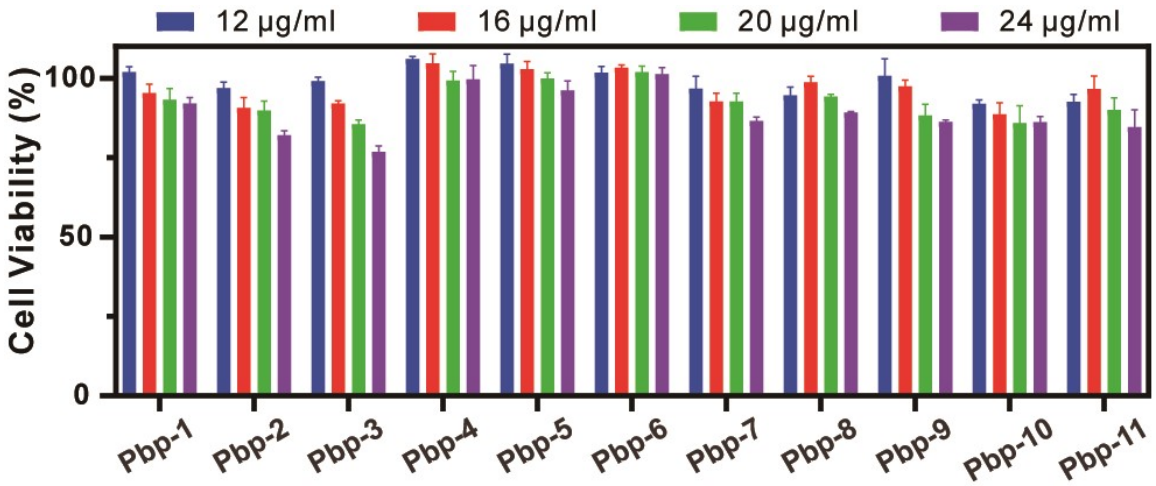


Figure S13. Cell viability of the Pbps 1-11 at different concentrations (12, 16, 20, 24 μg/mL) on 143B cells.

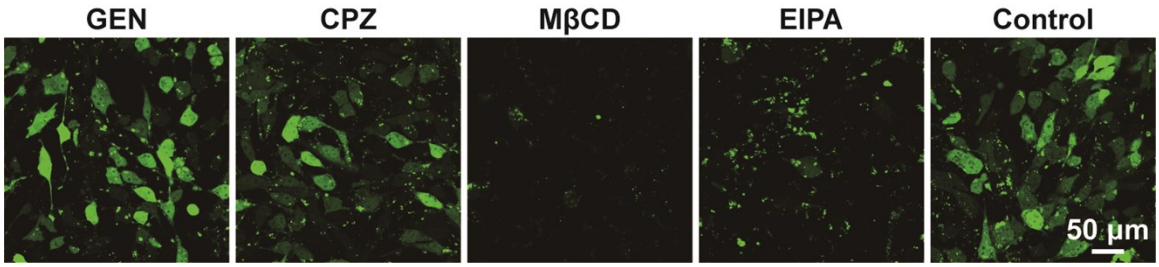


Figure S14. Confocal images of 143B cells pre-treated with different endocytosis inhibitors for 1 h before treatment with Pbp-4/EGFP complexes.

References

1 Q. Chen, D. Zhang, W. Zhang, H. Zhang, J. Zou, M. Chen, J. Li, Y. Yuan and R. Liu, *Nat. Commun.*, 2021, **12**, 562.

2 J. Zhang, D. A. Kissounko, S. E. Lee, S. H. Gellman and S. S. Stahl, *J. Am. Chem. Soc.*, 2009, **131**, 1589–1597.

3 R. Liu, X. Chen, S. H. Gellman and K. S. Masters, *J. Am. Chem. Soc.*, 2013, **135**, 16296–16299.

4 D. M. L. Goodgame, S. P. W. Hill, R. Lincoln, M. Quiros and D. J. Williams, *Polyhedron*, 1993, **12**, 2753–2762.

5 Y. Qian, F. Qi, Q. Chen, Q. Zhang, Z. Qiao, S. Zhang, T. Wei, Q. Yu, S. Yu, Z. Mao, C. Gao, Y. Ding, Y. Cheng, C. Jin, H. Xie and R. Liu, *ACS Appl. Mater. Interfaces*, 2018, **10**, 15395–15400.