Supporting Information

Unravelling the Endosomal Escape of pH-Responsive Nanoparticles using Split Luciferase Endosomal Escape Quantification Assay

Umeka Nayanathara,[a] Fan Yang,[a] Changhe Zhang,[a] Yufu Wang,[a] Bruna Rossi Herling,[b] Samuel A. Smith,[a] Maximilian A. Beach,[a] Angus P.R. Johnston,[,b] Georgina K. Such*[,a]*

[a] School of Chemistry, The University of Melbourne, Parkville, Victoria 3010, Australia

[b] Monash Institute of Pharmaceutical Sciences, Monash University, Parkville, Victoria 3052, Australia

Supplementary synthesis methods:

Synthesis of pyridyl disulfide ethyl methacrylate (PDSEMA)

Step 1: Synthesis of Hydroxy pyridyl disulfide

Aldrithol-2 (15 g, 0.068 mol) was dissolved in anhydrous methanol (75 mL) and glacial acetic acid (1 mL) was added while maintaining the nitrogen environment. To this mixture, 2-mercaptoethanol (2.4 mL, 0.034 mol) was added dropwise with continuous stirring at room temperature. After stirring for overnight, the excess solvent was evaporated to obtain the crude product as yellow oil. The crude mixture was purified by column chromatography over silica using ethyl acetate/hexane as the eluent. Excess Aldrithol-2 was first removed by 15%(v/v)

ethyl acetate/hexane mixture and then pure product was isolated using 40%(v/v) ethyl acetate/hexane as a colourless oil (5.1 g, 80% yield). ^{1}H NMR (400 MHz, $CDCl_3$): δ (ppm)), -8.47-8.42 (m, 1H, aromatic H), 7.59-7.54 (m, 1H, aromatic H), 7.41-7.35 (m, 1H, aromatic H), 7.20-7.00 (m, 1H, aromatic proton), 3.79-3.75 (t, 2H, -**CH_2**-OH), 2.92-2.89 (t, 2H, -S-**CH_2**-CH_2-OH) (**Figure S1)**.

Step 2: Synthesis of PDSEMA

Hydroxy pyridyl disulfide (2 g, 0.011 mol), TEA (1.51 g, 0.015 mol) and DMAP (0.26 g, 2.12 mmol) were dissolved in dry DCM (50 mL). The reaction mixture was cooled down to 0 ^oC. Methacrylic anhydride (2.3 g, 0.015 mol) was added dropwise with vigorous stirring under nitrogen. The reaction mixture was left to stir overnight at room temperature. The resulting crude reaction mixture was then washed with 0.1 M HCl (3×), water (3×) and brine (2×) respectively. The organic layer was then collected and dried over anhydrous $MgSO_4$, and excess solvent was evaporated under vacuum to obtain crude product as a yellow oil. The crude product was further purified by column chromatography using ethyl acetate/hexane as the eluent to obtain pure product as a yellow oil (2.1 g, 75% yield). ^{1}H NMR (400 MHz, $CDCl_3$): δ (ppm)), 8.47-8.42 (m, 1H, aromatic H), 7.70-7.66 (m, 1H, aromatic H), 7.64-7.61 (m, 1H, aromatic H), 7.10-7.00 (m, 1H, aromatic H), 6.10 (s, 1H, *cis* **CH_2**=CCH_3), 5.56 (s, 1H, *trans* **CH_2**=CCH_3), 4.38 (t, 2H, -S-CH_2-**CH_2**-O-), 3.07 (t, 2H, -S-**CH_2**-CH_2-O), 1.92 (s, 3H, C-**CH_3**) (**Figure S2**).

Supplementary results:

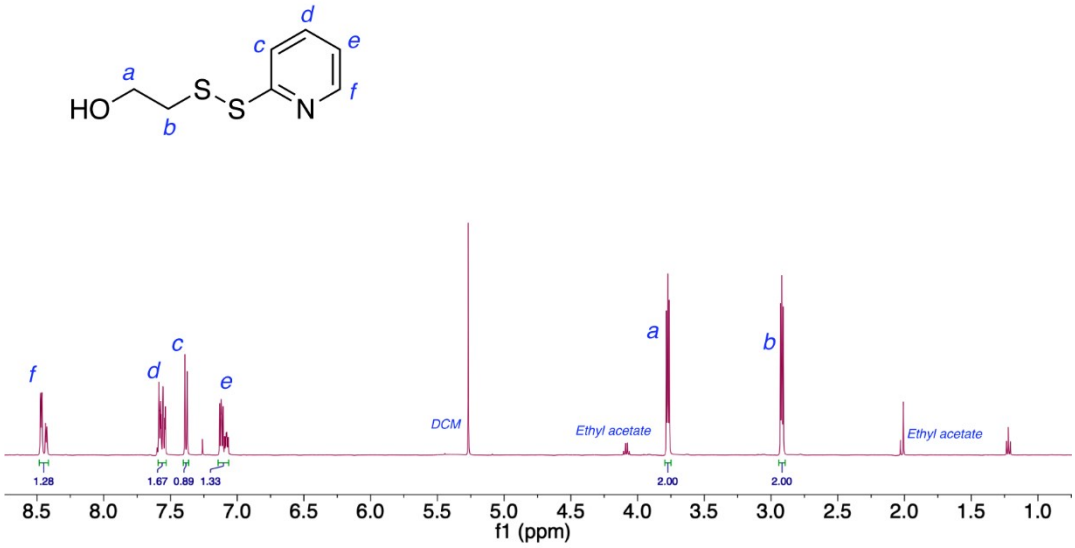


Figure S1. [1] H NMR spectrum of hydroxy pyridyl disulfide.

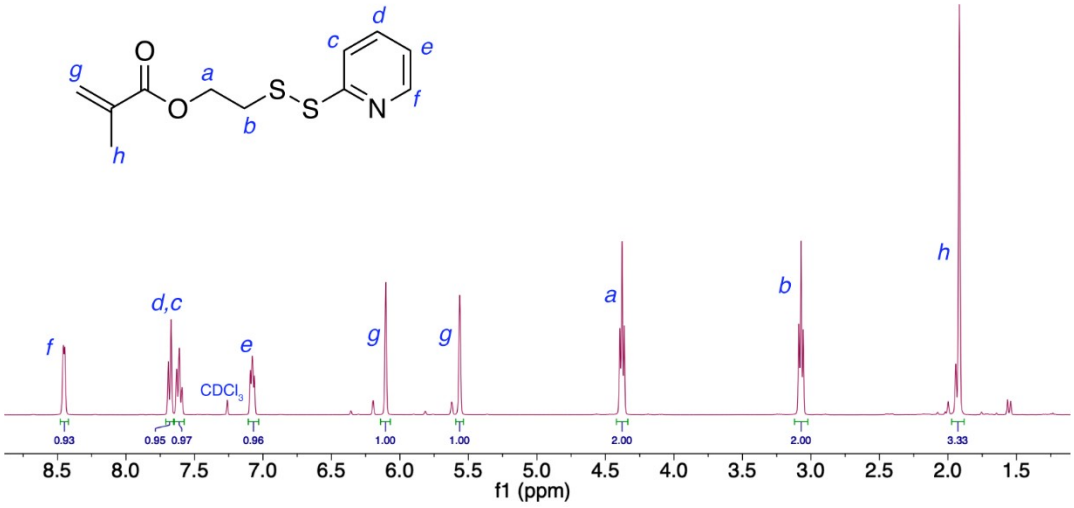


Figure S2. [1]H NMR spectrum of pyridyl disulfide ethyl methacrylate (PDSEMA).

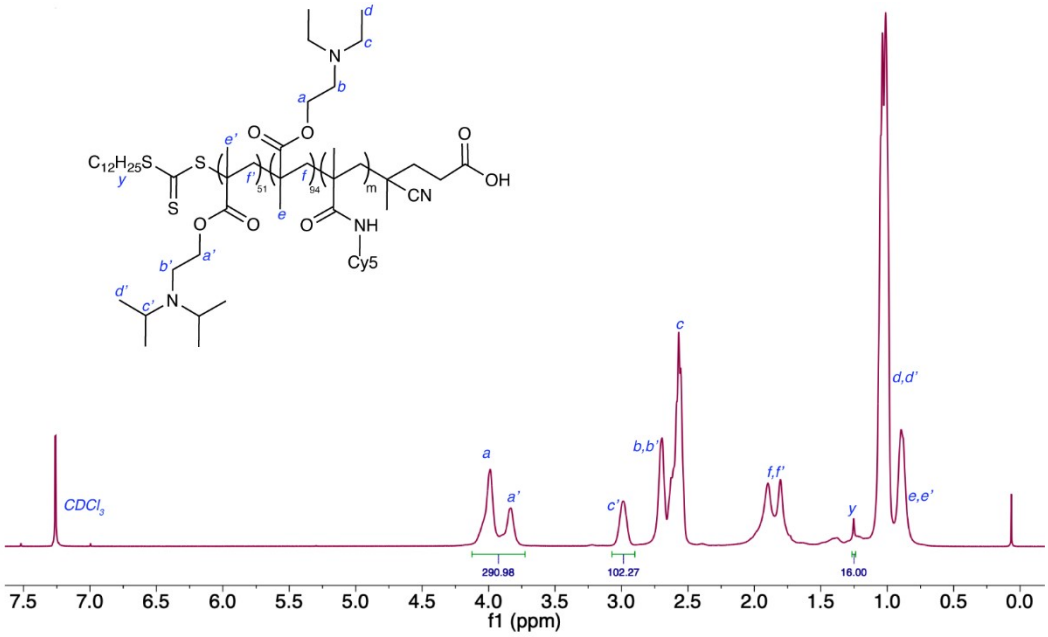


Figure S3. ^{1}H NMR spectrum of P(DEAEMA-*r*-DPAEMA-*r*-Cy5MA).

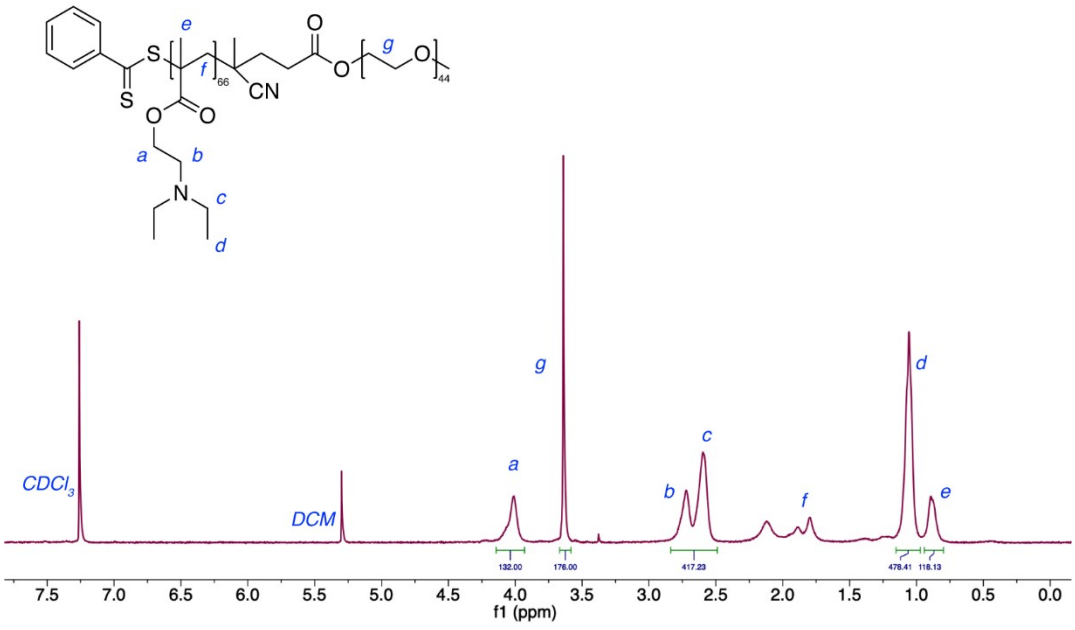


Figure S4. ^{1}H NMR spectrum of PDEAEMA-*b*-PEG.

Table S1: Material properties of core and shell polymers prepared via RAFT polymerization.

Polymer	Mn (kDa, ^{1}H NMR)	Mn[(a)] (kDa, SEC)	Đ [(b)]	Cy5 [(c)] molecules per polymer
P(DEAEMA-*r*-DPAEMA-*r*-Cy5MA)	30	24	1.03	0.007
PDEAEMA-*b*-PEG	15	12	1.17	-
P(DEAEMA-*r*-DPAEMA-*r*-PDSEMA)	40	16	1.12	-
P(DEAEMA-*r*-DPAEMA-*r*-HEMA)	42	43	1.19	-

[(a)] molecular weights were determined relative PMMA standards, and this may explain the discrepancy between the molecular weights calculated by SEC and NMR [(b)] dispersity of polymers determined by SEC. [(c)] determined *by* UV-Vis spectroscopy using a standard calibration curve of Cy5 amine in ethanol (Molar absorption coefficient of Cy5 amine at 646 nm - 148807 M^{-1} cm^{-1}).

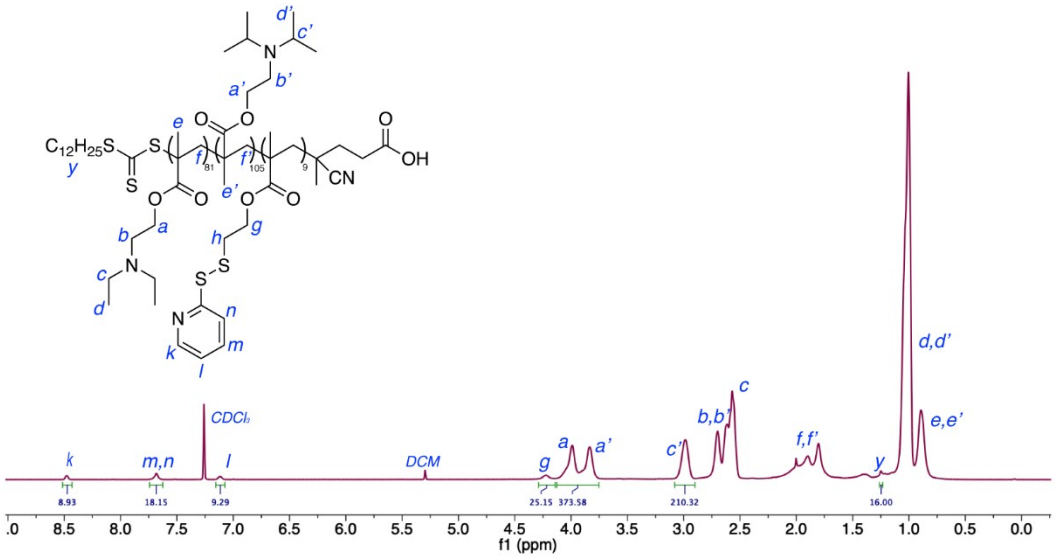


Figure S5. ^{1}H NMR spectrum of P(DEAEMA-*r*-DPAEMA-*r*-PDSEMA).

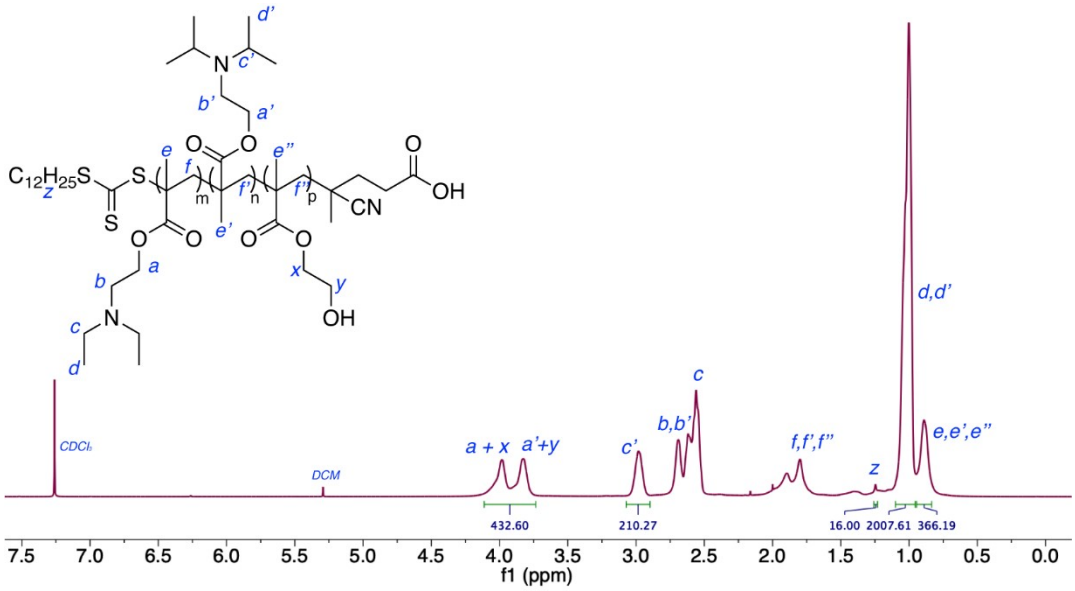


Figure S6. [1]H NMR spectrum of P(DEAEMA-*r*-DPAEMA-*r*-HEMA).

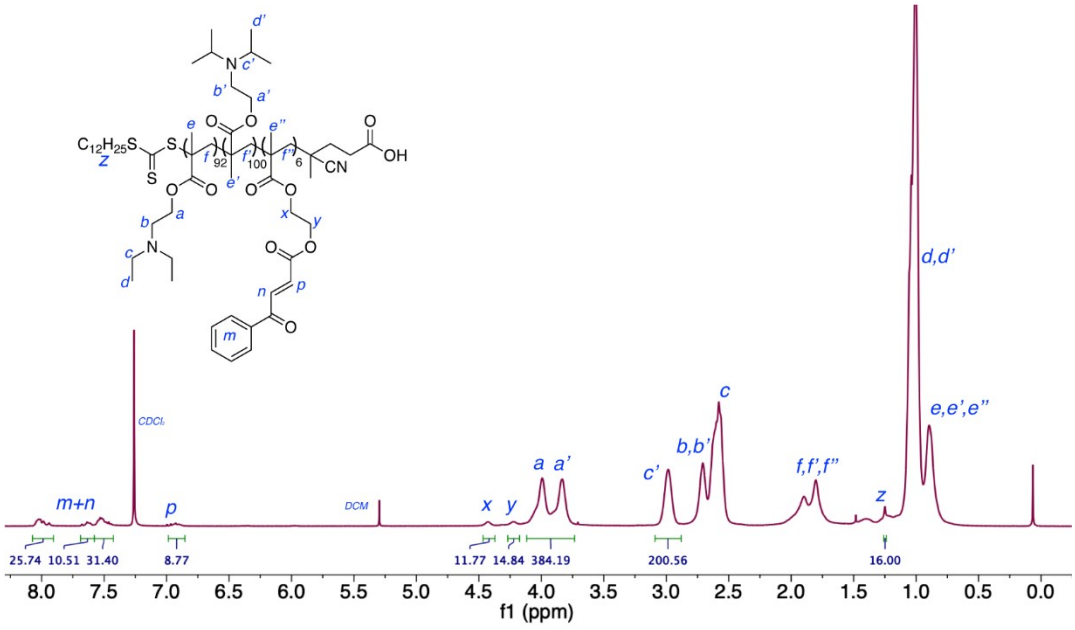
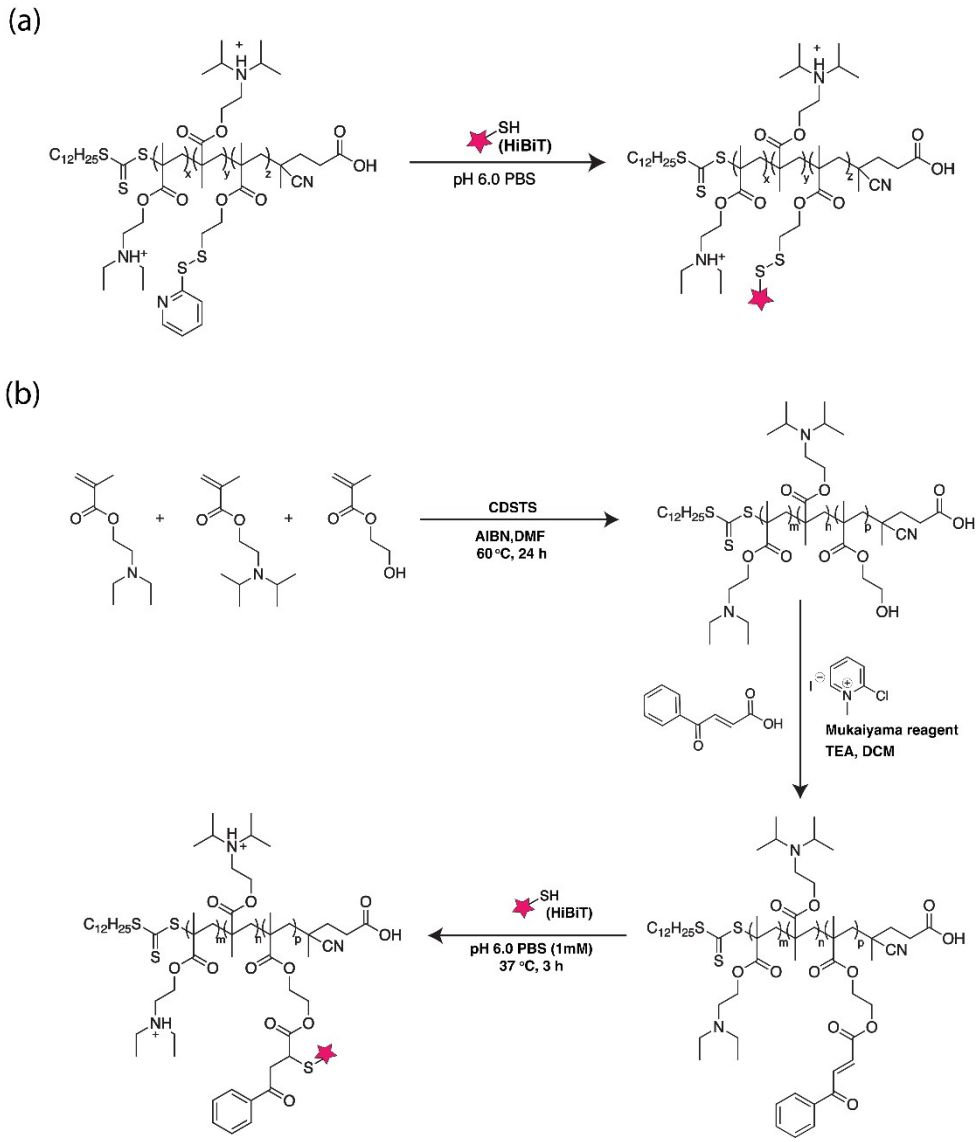


Figure S7. [1]H NMR spectrum of P(DEAEMA-*r*-DPAEMA-*r*- CaaEMA).



Scheme S1. General synthetic schemes for the synthesis of (a) cleavable HiBiT-conjugate via disulfide linkage and (b) non-cleavable HiBiT-conjugate via carbonylacrylic reagent functionalized thioether linkage .

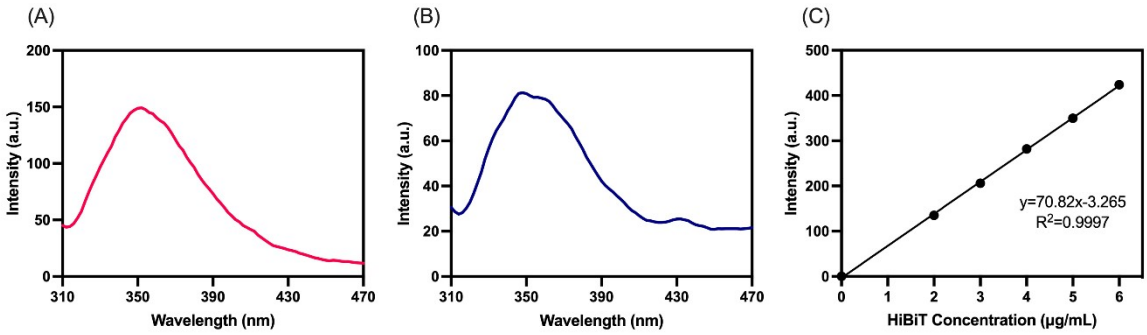


Figure S8. (a) Fluorescence emission spectrum of disulfide-linked HiBiT-conjugate in pH 6 PBS; (b) Fluorescence emission spectrum of thioether-linked HiBiT-conjugate in pH 6 PBS; (c) Calibration curve of HiBiT in pH 6.0 PBS (excitation wavelength – 273 nm and emission wavelength – 354 nm).

Table S2. Characterization of pH-responsive nanoparticles loaded with disulfide-linked and thioether-linked HiBiT-polymer conjugate

	DLS measurements		**NTA measurements**
	Mean size [a] (nm)	PDI [a]	Mean size [b] (nm)
NPs with disulfide-linked HiBiT conjugate	157 ± 7.85	0.11 ± 0.10	123 ± 2.40
NPs with thioether-linked HiBiT conjugate	148 ± 3.9	0.14 ± 0.07	112 ± 0.90

(a) Mean size and polydispersity (PDI) in pH 8 PBS at 25 ºC via DLS *(b)* Mean size in pH 8 PBS at 25 ºC via NTA.

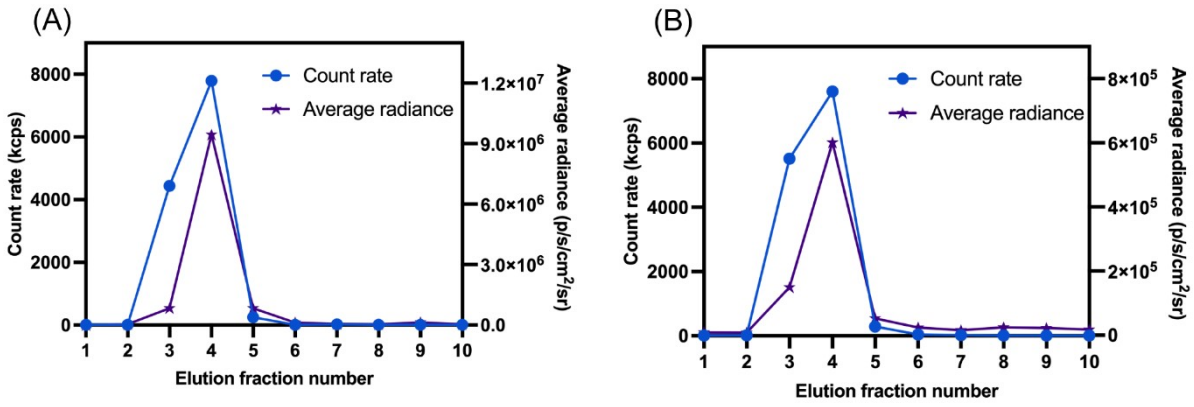


Figure S9. Analysis of eluted fractions from the Sepharose 6B column for nanoparticles loaded with (A) disulfide-linked and (B) thioether-linked HiBiT conjugates in pH 8 PBS. Luminescence signal is expressed as average radiance (★) in units of photons per second per centimetre squared per steradian (p/s/cm^2/sr). The count rate (●) was measured through DLS. Note: Neutral density filter (ND1 filter) was 100% for all the fractions.

Table S3. Physicochemical properties of nanoparticles via DLS before and after passing through the column. Size and polydispersity index (PDI) of nanoparticles were measured by DLS in pH 8.0 PBS at 25 ^{o}C. Data represents mean ± SD (n = 3).

	Before the column		**After the column**	
	Size (nm)	**PDI**	**Size (nm)**	**PDI**
NPs with disulfide-linked HiBiT conjugate	157	0.11	155	0.12
NPs with thioether-linked HiBiT conjugate	148	0.14	145	0.08

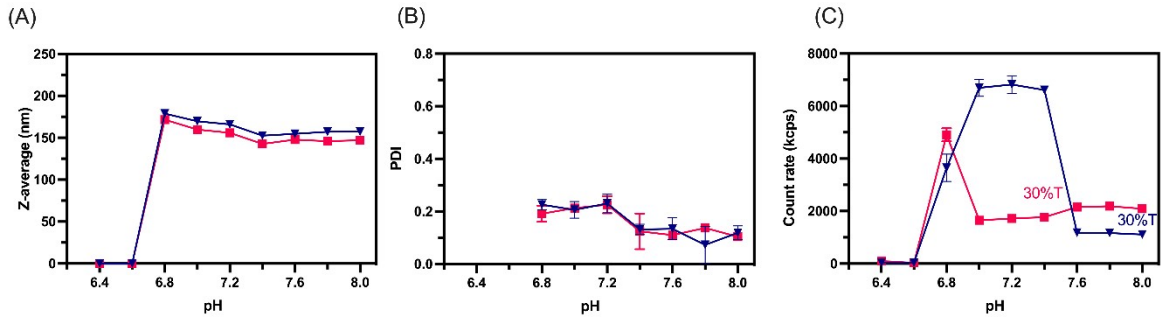


Figure S10: The pH disassembly profiles of each nanoparticle as a function of (A) Z-average vs pH, (B) polydispersity vs pH, and (C) count rate vs pH at 37 °C via DLS. Nanoparticles loaded with disulfide-linked HiBiT-conjugate are shown in red (■) and thioether-linked HiBiT-conjugate are shown in blue (▼). Note: For nanoparticles loaded with the disulfide-linked HiBiT conjugate, the neutral density filter was 30%T from pH 8.0 to 7.0. For nanoparticles loaded with the thioether-linked HiBiT conjugate, the neutral density filter was 30%T from pH 8.0 to 7.6. For all other points, the neutral density filter was 100%T.

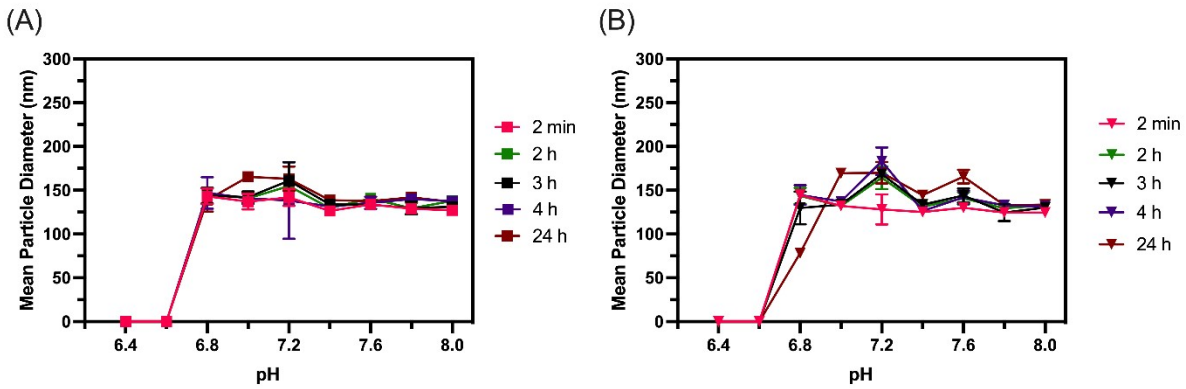


Figure S11: The pH disassembly profiles as a function of mean particle diameter vs pH at 37 °C for (A) nanoparticles loaded with disulfide-linked HiBiT-conjugate and (B) nanoparticles loaded with thioether-linked HiBiT-conjugate over different time intervals, 2 min, 2 h, 3 h, 4h and 24 h. Nanoparticles loaded with disulfide-linked HiBiT-conjugate are shown in square (■) and thioether-linked HiBiT-conjugate are shown in triangle (▼).

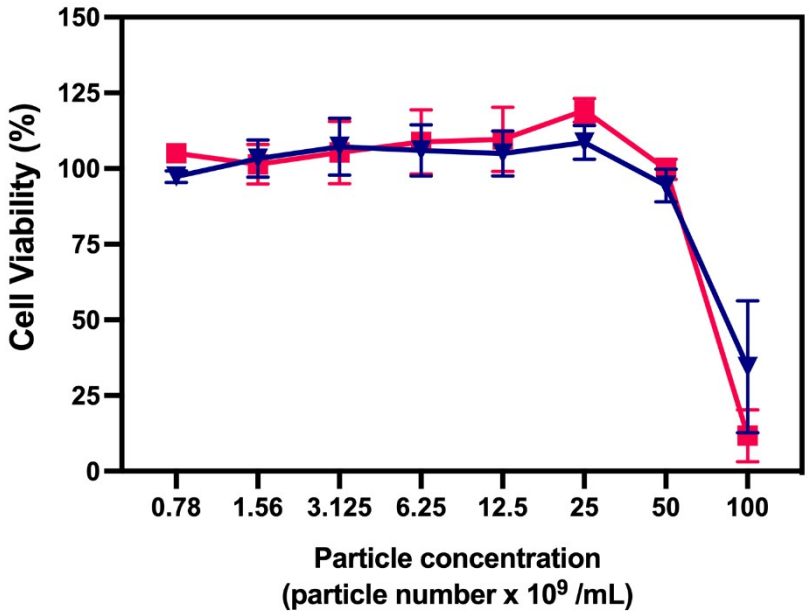


Figure S12. Cell viability of nanoparticles via alamarBlue assay in HEK293-LSA cells after 4 h incubation.

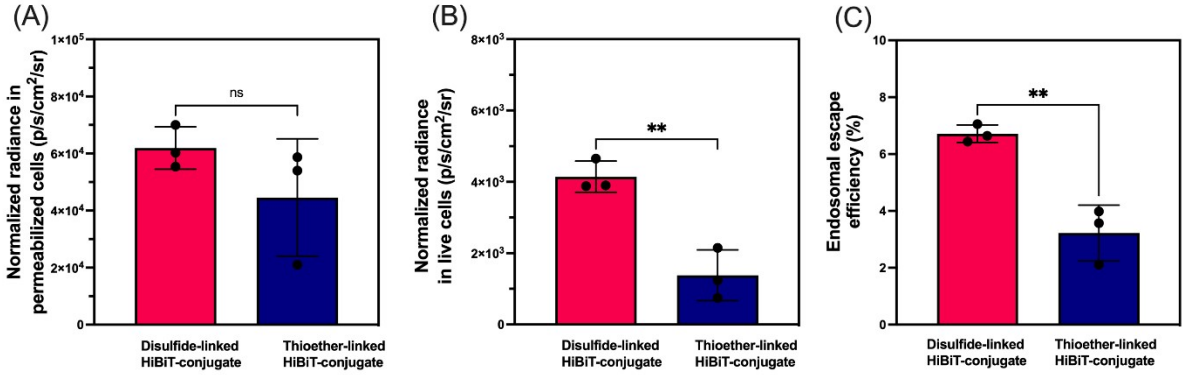


Figure S13. Endosomal escape efficiency of nanoparticles assessed via SLEEQ assay in HEK293-LSA cells for 4 h incubation. (A) Average radiance of total cellular association, (B) average radiance of cytosolic signal and (C) endosomal escape efficiency determined via dividing the cytosolic signal by total cellular association. Data represents mean ± SEM, n=3 independent experiments. Two-tailed unpaired *t*-test was performed to analyse the statistical difference between two nanoparticles (ns indicates $P > 0.05$, * indicates $P \leq 0.05$ ** indicates $p \leq 0.01$).