Supporting Information

Evaluating Endosomal Escape of Nanomaterials Using Split GFP with Caspase-3 as Cargo

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1. Materials and Instruments

Materials and Instruments. All chemicals were purchased from commercial sources, such as Thermo Fisher Scientific and Sigma Aldrich, and used without additional purification or modification. Azobisisobutyronitrile (AIBN) was purchased but was used after purification by recrystallization. Functionalization peptides were purchased from GenScript. ^{1}H and ^{13}C NMR spectra were recorded on a 400 MHz Bruker NMR spectrometer using the residual proton resonance of the solvent as the internal standards. Chemical shifts are reported in parts per million (ppm). Molecular weight of the polymers were estimated by gel permeation chromatography (GPC) in THF using poly(methyl methacrylate) (PMMA) standards with a refractive index detector. UV−Vis absorption spectra and fluorescence spectra were recorded on a Spectramax M5 spectrophotometer with clear or black 96-well plates, accordingly. Dynamic light scattering (DLS) measurements were performed using a Malvern Nanozetasizer. Thermal denaturation was measured using a CFX Connect Real-Time PCR detection system (BioRad Laboratories Inc.). Microscopy images provided by ZOE Fluorescent Cell Imager (Biorad) and Yokogawa Spinning Disc Confocal Microscope (Nikon) with image workup through ImageJ (FIJI). Flow cytometry experiments executed using BD DUAL LSR Fortessa Laser FACS and data analysis through FlowJo™ 10.

2. Additional methods/experimental

2.I Synthesis

2.I.i Random Copolymer synthesis –

- PEG-PDS random copolymer was synthesized as previously documented using commercially available polyethylene glycol monomethyl ether methacrylate (PEGMA) and synthesized pyridyl disulfide methacrylate (PDSMA) monomers in the presence of AIBN and a commercial RAFT reagent 4-cyano-4-[(dodecylsulfanylthiocarbonyl) sulfanyl]pentanoic acid.[1] Pyridyl disulfide methacrylate (PDSMA) was prepared using a previously reported procedure.[2] PDSMA (500 mg, 1.9 mmol), PEGMA (421 mg, 0.84 mmol) and RAFT reagent (14.85 mg, 0.0367 mmol) were dissolved in 800 µL dry THF in a 10 mL Schlenk flask and a catalytic amount of AIBN (1.2 mg, 0.0073 mmol) in 200 µL dry THF was added to that. The solution was degassed by performing three freeze−pump−thaw cycles with an argon inflow into the reaction. The reaction flask was tightly sealed and placed in a preheated oil bath at 70 °C with overnight stirring. The next day, the polymerization was quenched, and the polymer was purified by precipitation 3X in diethyl ether. Finally, the product was dissolved in DCM, evaporated and dried under high vacuum to get a highly viscous yellow polymer. GPC (DMF) Mn: 19.8 kDa, PDI: 1.20. The relative ratio between PEGMA and PDSMA were calculated from ^{1}H NMR considering the integration of peaks corresponding end group methyl of PEGMA at 3.3 ppm and aromatic peak of PDSMA at 8.5 ppm which was found to be PEGMA:PDSMA = 32:68. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.45, 7.66, 7.26, 7.09, 4.21, 4.07, 3.63, 3.37, 3.01, 1.91,1.82, 1.05, 0.87. ^{13}C NMR (101 MHz, $CDCl_3$) δ 159.4, 149.87, 137.1, 121.01, 119.9, 77.04, 71.94, 70.68, 68.47, 62.68, 59.94, 44.80, 36.87, 31.83, 29.59, 22.75.
- PEG-PDS-Bu random copolymer (was synthesized similarly. PDSMA (200 mg, 0.7843 mmol), PEGMA (314 mg, 0.628 mmol), butyl methacrylate (22.30 mg, 0.1568 mmol) and RAFT (8.657 mg, 0.02146 mmol) reagent were dissolved in 600 µL dry THF in a 10 mL Schlenk flask and a catalytic amount of AIBN (0.704 mg, 0.00428 mmol) in 100 µL dry THF was added to that. The solution was degassed by performing three freeze−pump−thaw cycles with an argon inflow into the reaction. The reaction flask was tightly sealed and placed in a preheated oil bath at 70 °C with overnight stirring. The next day, the polymerization was quenched, and the polymer was purified by precipitation 3X in diethyl ether. Finally, the product was dissolved in DCM, evaporated and dried under high vacuum to get a highly viscous yellow polymer. GPC (THF) Mn: 14.2 kDa, PDI: 1.25. The relative ratio between PEGMA, PDSMA and butyl methacrylate was calculated from ^{1}H NMR considering the integration of peaks corresponding end group methyl of PEGMA at 3.3 ppm, aromatic peak of PDSMA at 8.5 ppm and α proton of butyl methacrylate at 3.8 ppm which were found to be PEGMA:PDSMA:butyl methacrylate = 45:38:17. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.47, 7.68, 7.26, 7.12, 4.22, 4.07, 3.92, 3.64, 3.37, 3.03, 1.93, 1.81, 1.7, 1.6, 1.37, 1.03, 0.92. ^{13}C NMR (101 MHz, $CDCl_3$) δ150.09, 137.56, 121.43, 120.41, 77.1, 71.33, 70.96, 68.78, 63.01, 59.51, 49.41, 45.20, 37.18, 21.30.
- PEG-PDS-IPA random copolymer was synthesized similarly as previously reported.[3] PDSMA (200 mg, 0.7843 mmol), PEGMA (314 mg, 0.628 mmol), 2-(Diisopropylamino) ethyl methacrylate (33.46 mg, 0.1568 mmol) and RAFT (9.754 mg, 0.02146 mmol) reagent were dissolved in 600 µL dry THF in a 10 mL Schlenk flask and a catalytic amount of AIBN (0.793 mg, 0.00428 mmol) in 100 µL dry THF was added to that. The solution was degassed by performing three freeze−pump−thaw cycles with an argon inflow into the reaction. The reaction flask was tightly sealed and placed in a preheated oil bath at 70 °C with overnight stirring. The next day, the polymerization was quenched, and the polymer was purified by precipitation 3X in diethyl ether. Finally, the product was dissolved in DCM, evaporated and dried under high vacuum to get a highly viscous yellow polymer. GPC (THF) Mn: 14.3 kDa, PDI: 1.26. The relative ratio between PEGMA, PDSMA and butyl methacrylate was calculated from ^{1}H NMR considering the integration of peaks corresponding end group methyl of PEGMA at 3.3 ppm, aromatic peak of PDSMA at 8.5 ppm and 2-(Diisopropylamino) ethyl methacrylate proton at 2.6 ppm which were found to be

PEGMA:PDSMA: 2-(Diisopropylamino) ethyl methacrylate = 49:41:10. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.46, 7.68, 7.26, 7.11, 4.21, 4.07, 3.64, 3.37, 3.03, 2.61, 1.84, 1.25, 0.99,0.88. ^{13}C NMR (101 MHz, $CDCl_3$) δ 149.82, 137.18, 120.90, 119.82, 77.03, 71.84, 70.55, 68.43, 59.05, 44.88, 36.96, 30.10, 22.63, 19.35, 13.81.

2.I.ii Block Copolymer synthesis –

- PEG-b-PDS block copolymer was synthesized using a commercial PEG-RAFT reagent, poly(ethylene glycol) methyl ether (4-cyano-4-pentanoate dodecyl trithiocarbonate), with an average molecular weight of 5400 g/mol (200 mg, 0.037mmol), and PDSMA (94.57 mg, 0.37mmol). The two monomers were combined and dissolved in 200 µL dry THF in a 10 mL Schlenk flask using sonication. AIBN (1.22 mg, 0.0074 mmol) was then added to the mixture and the solution degassed by performing three freeze−pump−thaw cycles with an argon inflow into the reaction. The reaction vessel was sealed and placed in a preheated oil bath at 70 °C for 6 h. The product polymer P1 was then purified by precipitation in cold ether (20 mL) and dried under vacuum to yield the block copolymer. Yield: 88%. GPC (DMF) Mn: 5.4 KDa, Mw: 7.0KDa, PD: 1.31. ^{1}H NMR (400 MHz, CDCl3) δ 8.45, 7.65, 7.28, 7.08, 4.22, 3.82, 3.81, 3.65, 3.48, 3.46, 3.38, 3.02, 2.11, 1.94, 1.93, 1.85, 1.32, 1.24, 1.19, 1.07, 0.92, 0.90, 0.89. The molar ratio between PEG5400 and the two monomers was determined by the integration of end group methyl proton from PEG at 3.3 ppm, aromatic proton from PDSMA at 8.5 ppm which found to be (PEG_{5400}:PDS) = (1:12). ^{13}C NMR (101 MHz, CDCl3) δ 159.58, 159.42, 149.76, 137.18, 120.95, 119.90, 119.83, 77.38, 77.06, 76.75, 72.57, 71.94, 70.57, 68.99, 61.71, 59.04, 44.84, 36.95, 36.83, 31.90, 29.62, 29.55, 29.44, 29.33, 29.10, 28.96, 22.68, 18.88, 14.14.
- PEG-b-PDS-Bu block copolymer (PbPB) was synthesized similarly. A mixture of PEG-RAFT (200 mg, 0.037mmol), PDSMA (85.12 mg, 0.33mmol), and butyl methacrylate (BUMA) (5.27 mg, 0.037mmol) were dissolved in 200 µL of dry THF in a 10 mL Schlenk flask using sonication. AIBN (1.22 mg, 0.0074 mmol) was then added to the mixture and the solution degassed by performing three freeze−pump−thaw cycles with an argon inflow into the reaction. The reaction vessel was sealed and placed in a preheated oil bath at 70 °C for 6 h. The product polymer P1 was then purified by precipitation in cold ether (20 mL) and dried under vacuum to yield the block copolymer. Yield: 81%. GPC (THF) Mn: 5.7 KDa, Mw: 7.1 KDa, PD: 1.24. ^{1}H NMR (400 MHz, CDCl3) δ 8.44, 7.67, 7.65, 7.62, 7.26, 7.08, 7.07, 4.21, 3.91, 3.81, 3.64, 3.47, 3.46, 3.37, 3.02, 3.00, 1.94, 1.92, 1.87, 1.81, 1.72, 1.31, 1.25, 1.23, 1.23, 1.18, 1.06, 0.91, 0.89, 0.87, 0.87, 0.86.The molar ratio between PEG5400 and the two monomers were determined by the integration of end group methyl proton from PEG at 3.3 ppm, aromatic proton from PDSMA at 8.5 ppm and carbon next to ester group from BUMA at 3.9 ppm and found to be (PEG_{5400}:PDS:BU) = (1:11:1). ^{13}C NMR (101 MHz, CDCl3) δ 149.77, 137.18, 120.96, 119.91, 77.35, 77.03, 76.72, 72.59, 71.95, 70.58, 61.73, 59.05, 44.84, 31.91, 29.62, 29.34, 22.69, 14.15.

2.II NG characterization

2.II.i Nanogel size characterization – Purified nanogels were diluted to ~0.5-1 mg/mL in water and subsequently filtered to remove dust using a 0.45 µm syringe filter. After filtration, samples were subjected to DLS characterization using a Malvern Nanozetasizer operating at 633 nm. For each sample, three readings with 10 runs each were taken and averaged.

2.II.ii Nanogel mediated protein encapsulation and release – Purified nanogels were removed from dialysis and volumes normalized. To visualize protein release, 30 µL of the nanogel solution was incubated with either 10 µL of 1M DTT or autoclaved water and left for 15 minutes at RT. Next, 10 µL of SDS-PAGE 3X dye (with reductant) was added to the DTT-NG sample and reductant free SDS-PAGE 3X dye was added to the water-NG sample. The samples were immediately boiled at 95 °C for ~5 minutes and then added to a 16% SDS-PAGE and electrophoresis was executed at 175V for 60 minutes. Control protein samples were prepared using 30 µL of 10 µM protein and 10 µL of SDS-PAGE 3X dye, subsequently adding 10, 5, 2.5 and 1.25 µL to the gel, respectively. To compare encapsulation efficiencies, only when ran on the same gel, full-length $C3^{KO}$-11 band intensities of different concentrations were made into a calibration curve and compared to NG-released $C3^{KO}$-11 using Image Lab™ Software.

2.II.iii Nanogel visualization by microscopy – All microscopy was executed using live cells to avoid artefacts due to cell fixation. Cell culture dishes were seeded with cells at a density of 100,000 cells/mL and left to adhere for 24 hours. Purified NG were then added and incubated for the appropriate amount of time. After time elapsed, NG were removed and cells were washed twice with 1X PBS, pH 7.4 to remove any non-endocytosed NG. Cells were then treated with stains such as NucBlue™ stain ReadyProbe™ (Thermo) and Lysotracker Green. After incubation with the stains for 1 hour, media was removed and cells washed again. Finally, cells were left in Live Cell Imaging Solution (with 10% FBS, Thermo) for microscopy analysis. For Cy-labeled proteins, briefly, $C3^{KO}$-11 were labeled with two equivalents of Cy3-NHS ester (Lumiprobe) in a sodium bicarbonate buffer (pH 8.5) for three hours at room temperature. Free dye was removed via purification using NAP5 columns (GE Healthcare) before NG formation. For Cy-labeled polymers, polymers were covalently labeled using Cy3 labeled RAFT (chain transfer agent) and purified normally.

2.II.iv Immunoblotting – Lysates were assayed for the total protein concentration by bicinchoninic acid assay (Pierce). Lysates were then normalized to identical protein concentration and SDS-PAGE samples were immediately prepared and boiled at 95 °C for ~10 minutes. 20 µL of lysate samples were loaded into a 16% SDS-PAGE and electrophoresis was executed at 175V for 70 minutes. Samples were then transferred to a PVDF membrane (Millipore) at 100V for 140 minutes, with two ice block changes. After transfer, blots were blocked using OneBlock Western-CL blocking buffer (Genesee Scientific) for 60 minutes at room temperature and then cut into appropriate molecular weight ranges to probe for specific proteins. Blots were incubated with the primary antibody overnight at 4 °C with constant rotation. All primary antibodies used were purchased from cell-signaling technologies using a 1:1,000 dilution including anti-alpha tubulin (#2144S), anti-casp-3 (#9662S), anti-FL PARP(#9532S), anti-cleaved PARP (#9541S) and anti-His (#12698S). Blots were then washed with 1X TBST 3X for 10 minutes each and then probed with a secondary goat anti-rabbit-HRP antibody (#20-303, Genesee Scientific) at 1:20,000 dilution for 1 hour at room temperature. Blots were then washed again with 1X TBST 3X for 10 minutes each followed by incubation with SuperSignal™ West Duration Substrate (#34075, Thermo Fisher) for 2-3 minutes at room temperature and imaged using the ChemiDoc MP Imaging System (BioRad). Blots were quantified using the ImageJ and normalized to the alpha-tubulin loading control.

2.III Mesoporous silica - MSi

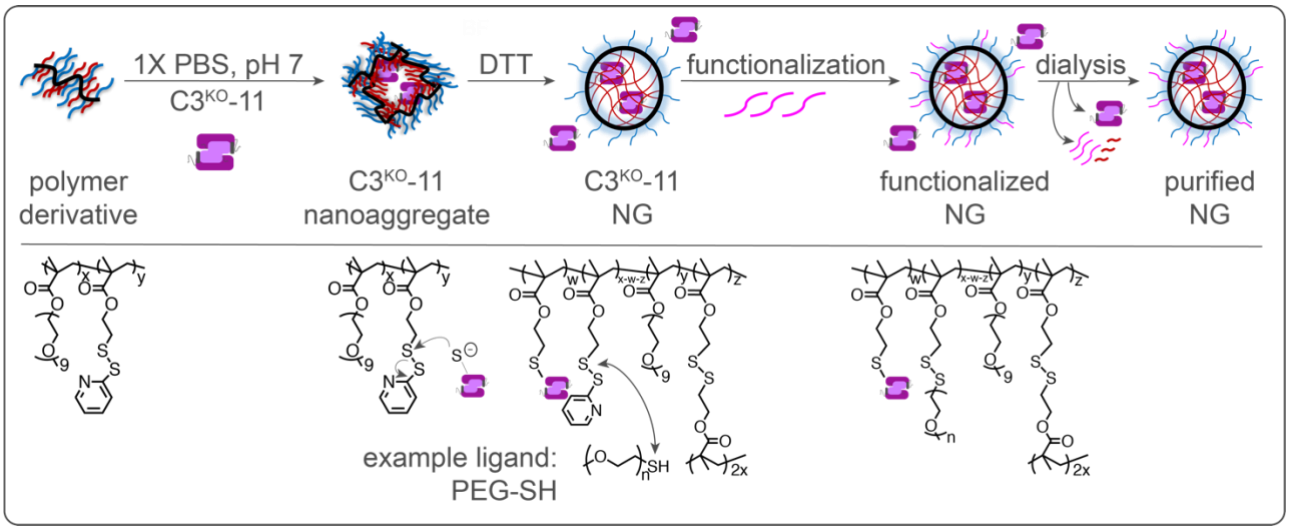
2.III.i Synthesis of amine functionalized mesoporous silica (MSi-NH_2) particles – MSi particles were prepared according to a published procedure.[4] Further, the MSi particles were functionalized with amine groups through a silica coupling reaction according to our previous work.[5] Typically, 400 mg MSi was dispersed in a mixture of 20 mL MeOH, 5 mL water and 1 mL ammonium hydroxide. Then, 0.5 mL (3-Aminopropyl)trimethoxysilane (APS) was added dropwise under stirring. The reaction was performed at 60 °C for 4 h. The product was harvested by centrifugation and purified by washing with water and methanol for 5 times each. The final MSi-NH_2 was dried under high vacuum until constant weight.

2.III.ii MSi-NH_2 linker synthesis –The details for synthesis and characterization of the linkers (**1&2**) were reported in our previous work. [5,6]

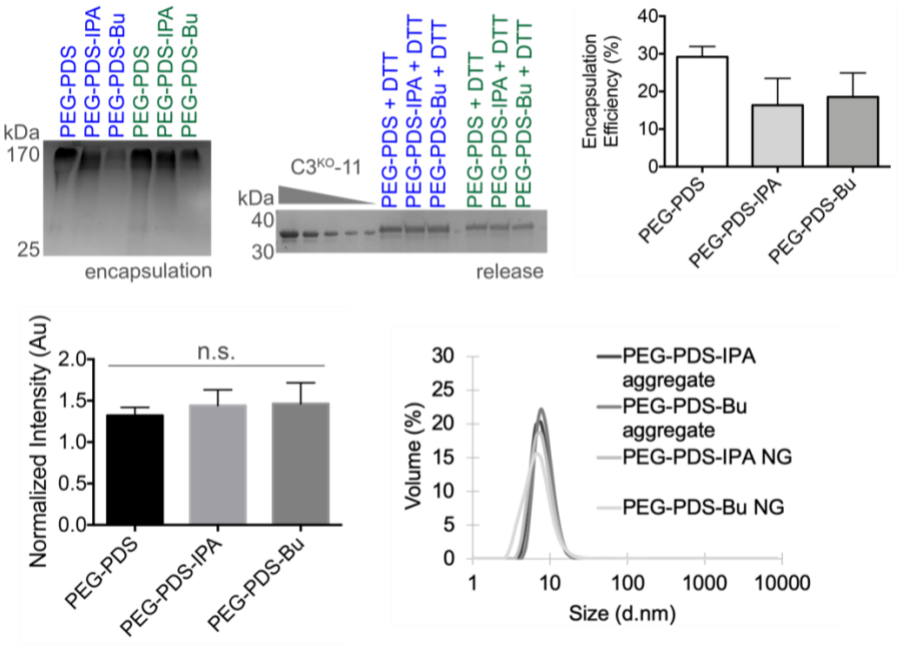
2.III.iii Protein-MSi complexation – Proteins and MSi-NH_2 were mixed with a mass ratio of 1:5 under water for 5 mins and then used fresh.

3. Supplemental Figures (S#)

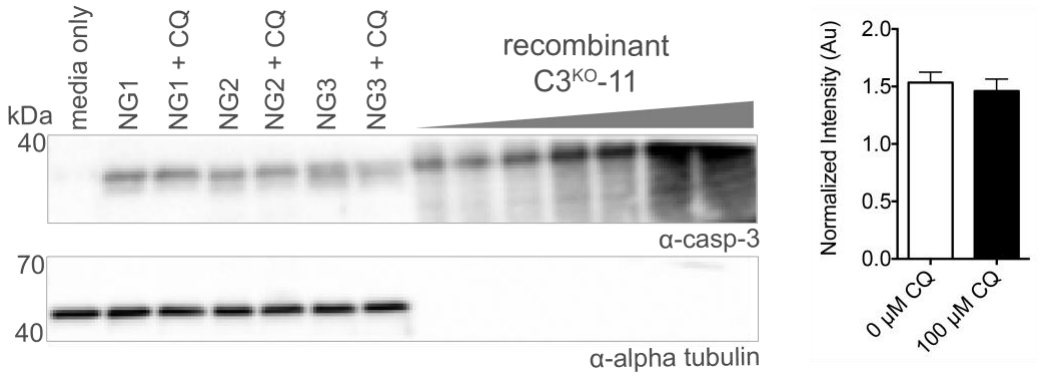
3.I Figure S1. C3-11 NG formation: schematic and structural description.



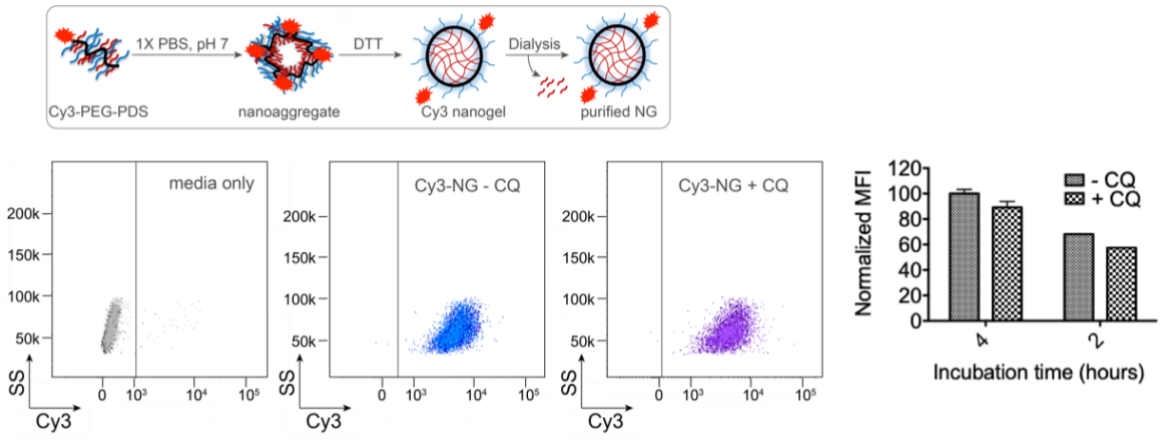
3.II Figure S2. Random copolymer NG encapsulation and release. Top: PEG-PDS-IPA and PEG-PDS-Bu encapsulated (0 mM DTT) and released (150 mM DTT) C3^{KO}-11 comparably to PEG-PDS, visualized by Coomassie stained SDS-PAGE (*in vitro* experiment). Encapsulation efficiencies for all polymers were compared using a standard curve of free C3^{KO}-11, quantified using Image Lab^{TM} Software. Bottom: The resultant NG intracellularly delivered similar amounts of C3^{KO}-11 overall by western blot. Finally, the resultant NG size of all polymers were monodispersed, characterized by DLS as described in the methods, with polydispersity indexes 0.3 ± 0.04, 0.4 ± 0.2 and 0.5 ± 0.3 for PEG-PDS, PEG-PDS-IPA and PEG-PDS-Bu, respectively.



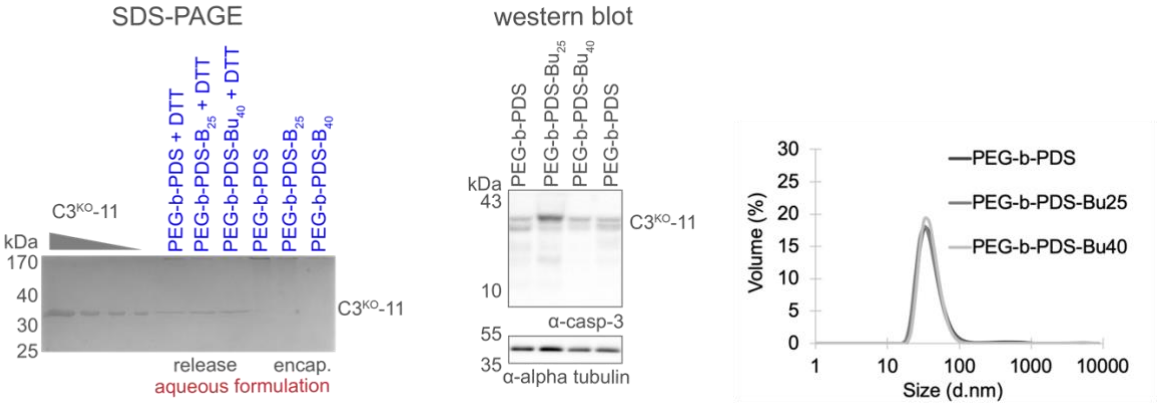
3.V Figure S3. Chloroquine does not increase total amount of protein delivered. C3^{KO}-11 NG were added to HEKs1-10 cells in the presence or absence of CQ. Cells were then lysed and analyzed by western blot (band intensity of C3^{KO}-11 was normalized to alpha tubulin) to determine the total amount of protein delivered intracellularly. These data demonstrated that CQ did not significantly influence the amount of total protein delivered.



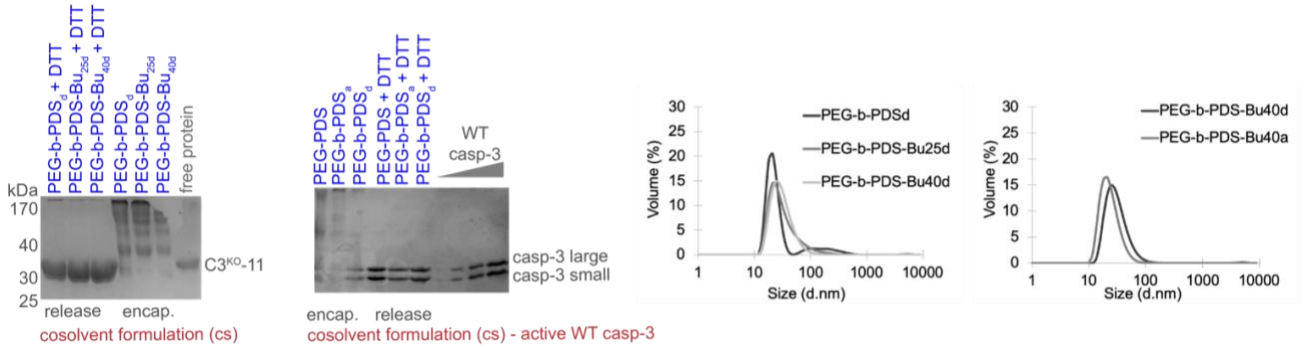
3.V Figure S4. Chloroquine does not increase total amount of polymer delivered. Cy3 labeled PEG-PDS chains were used to make fluorescently labeled nanogels, containing C3^{KO}-11. Cy3-NG were added to cells in the presence or absence of CQ and incubated for three hours. Media was removed and cells were washed twice with 1X PBS, pH 7.4 to remove any non-endocytosed NG. The amount of NG uptaken was analyzed by flow cytometry. These data demonstrated that CQ did not significantly influence the amount of total Cy3-NG internalized.



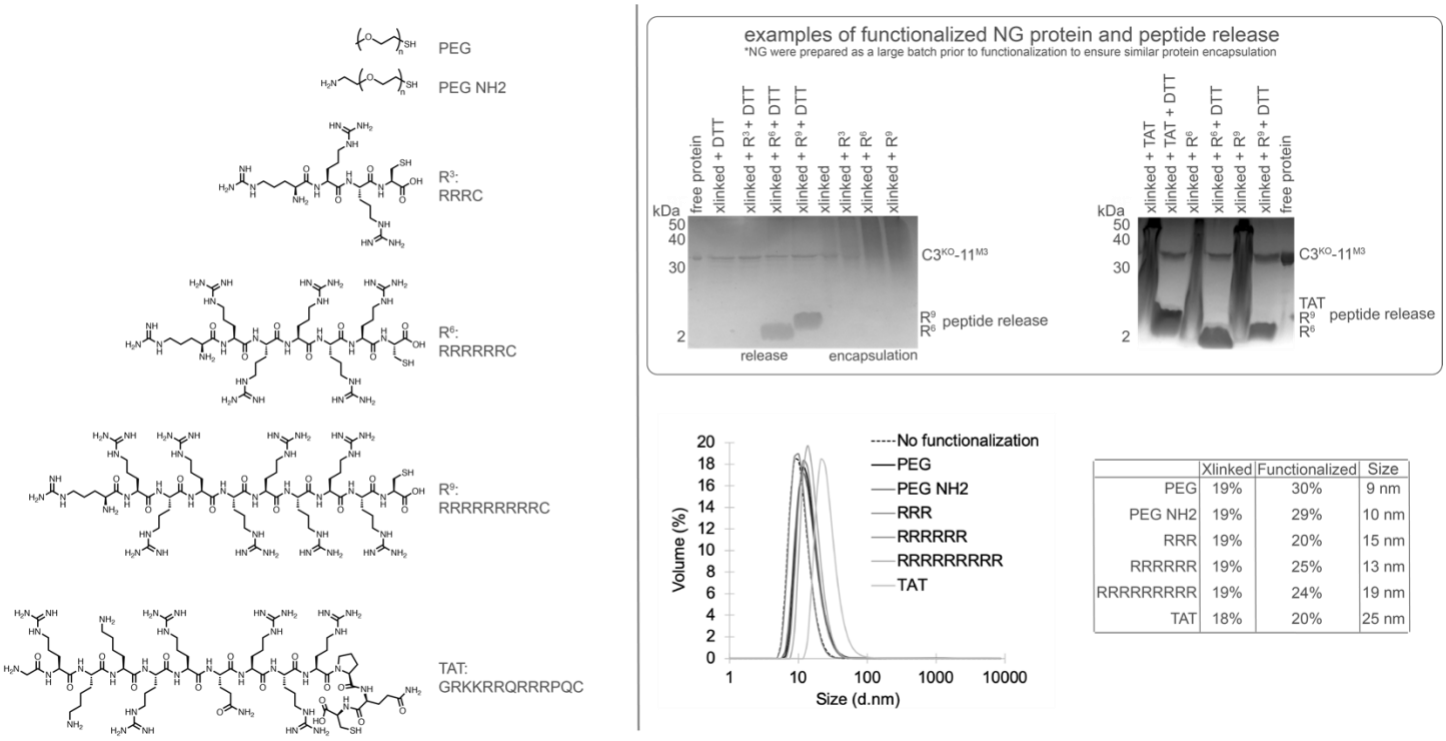
3.V Figure S5. Aqueous block copolymer NG encapsulation, release and delivery. Left: PEG-b-PDS and PEG-b-PDS-Bu$_{25,40}$ encapsulated (0 mM DTT) and released (150 mM DTT) a small amount of C3^{KO}-11, visualized by coomassie stained SDS-PAGE (*in vitro* experiment). Middle: PEG-b-PDS and PEG-b-PDS-Bu$_{25,40}$ mediated C3^{KO}-11 delivery to HEKs1-10 cells, visualized by western blot. Right: The resultant NG size of all block polymers were monodispersed, characterized by DLS as described in the methods, with polydispersity indexes 0.2 ± 0.01, 0.2 ± 0.01 and 0.2 ± 0.03 for PEG-b-PDS, PEG-b-PDS-Bu$_{25}$ and PEG-b-PDS-Bu$_{40}$ respectively.



3.VI Figure S6. Co-solvent block copolymer NG encapsulation and release. Left gel: Block copolymers encapsulated (0 mM DTT) and released (150 mM DTT) a significantly higher amount of $C3^{KO}$-11 compared to the aqueous formulation, visualized by coomassie stained SDS-PAGE (*in vitro* experiment). Right gel: Block copolymers also demonstrated encapsulation (0 mM DTT) and release (150 mM DTT) of WT casp-3. Right: The resultant NG size of all block polymers were monodispersed, characterized by DLS as described in the methods, with polydispersity indexes 0.3 ± 0.03, 0.3 ± 0.01, 0.3 ± 0.02 and 0.3 ± 0.01 for PEG-b-PDS_d, PEG-b-PDS-Bu_{25d}, PEG-b-PDS-Bu_{40d}, PEG-b-PDS-Bu_{40a} respectively.

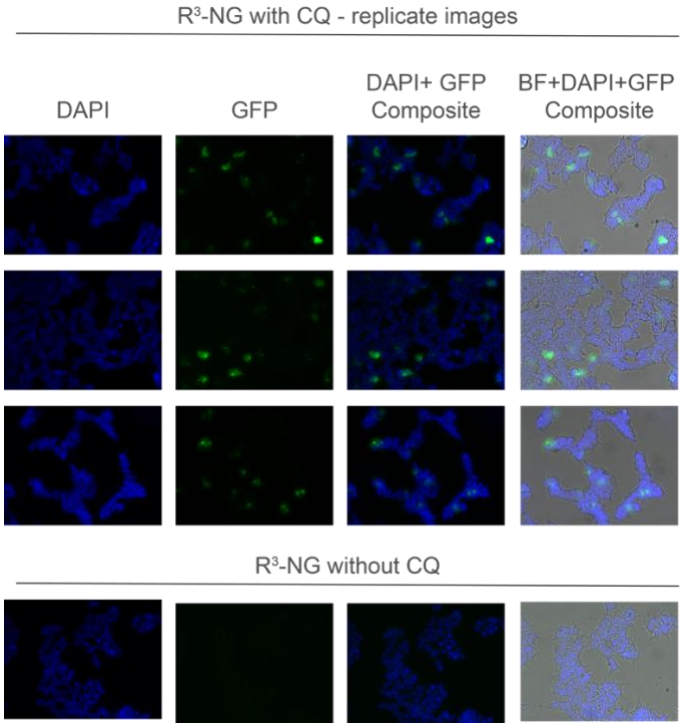


3.VII Figure S7. Cationic PEG-PDS Functionalized NG characterization. Left: Structures and abbreviations of cationic peptides used for NG functionalization. Right: Visualization of surface functionalized peptide release (150 mM DTT) of $C3^{KO}$-11 NG and resultant sizes after crosslinking and functionalization, characterized by DLS as described in the methods, with polydispersity indexes 0.2 ± 0.01, 0.4 ± 0.03, 0.4 ± 0.02 and 0.2 ± 0.1 for RRR, RRRRRR, RRRRRRRRR and TAT respectively.

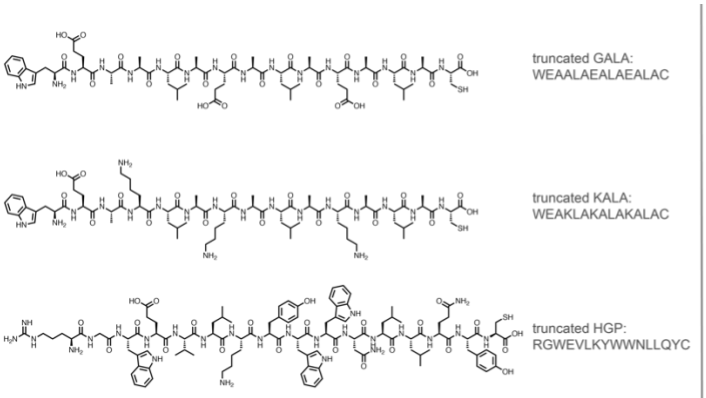


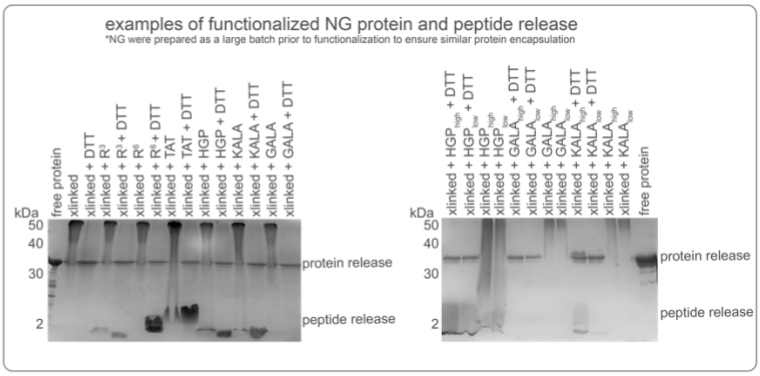
	Xlinked	Functionalized	Size
PEG	19%	30%	9 nm
PEG NH2	19%	29%	10 nm
RRR	19%	20%	15 nm
RRRRRR	19%	25%	13 nm
RRRRRRRRR	19%	24%	19 nm
TAT	18%	20%	25 nm

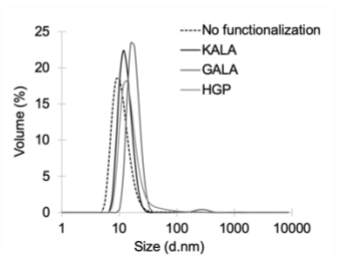
3.VIII Figure S8. Cationic PEG-PDS Functionalized NG imaging in HEKS1-10. HEKs1-10 cells (seeded in glass-bottom imaging dishes) were incubated with cationic peptide functionalized NG, in the absence or presence of CQ. Cells were incubated with NG for 24 hours followed by nucleus staining (NucBlue^{TM}) and subsequent imaging using 405 nm and 488 nm laser lines.



3.IX Figure S9. pH-responsive PEG-PDS Functionalized NG characterization. Left: Structures and abbreviations of pH-sensitive peptides used for NG functionalization. Right: Visualization of surface functionalized peptide release (150 mM DTT) of C3^{KO}-11 NG and resultant sizes after crosslinking and functionalization, characterized by DLS as described in the methods, with polydispersity indexes 0.4 ± 0.1, 0.3 ± 0.2 and 0.5 ± 0.02 for KALA, GALA and HGP respectively.

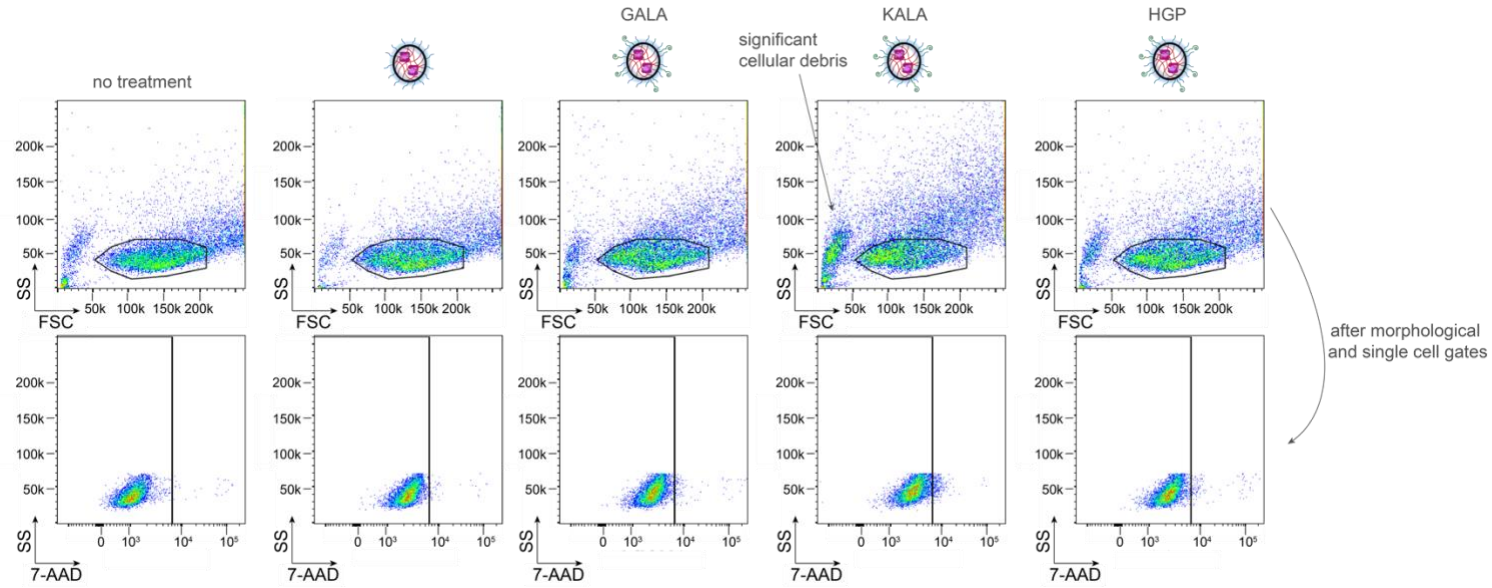




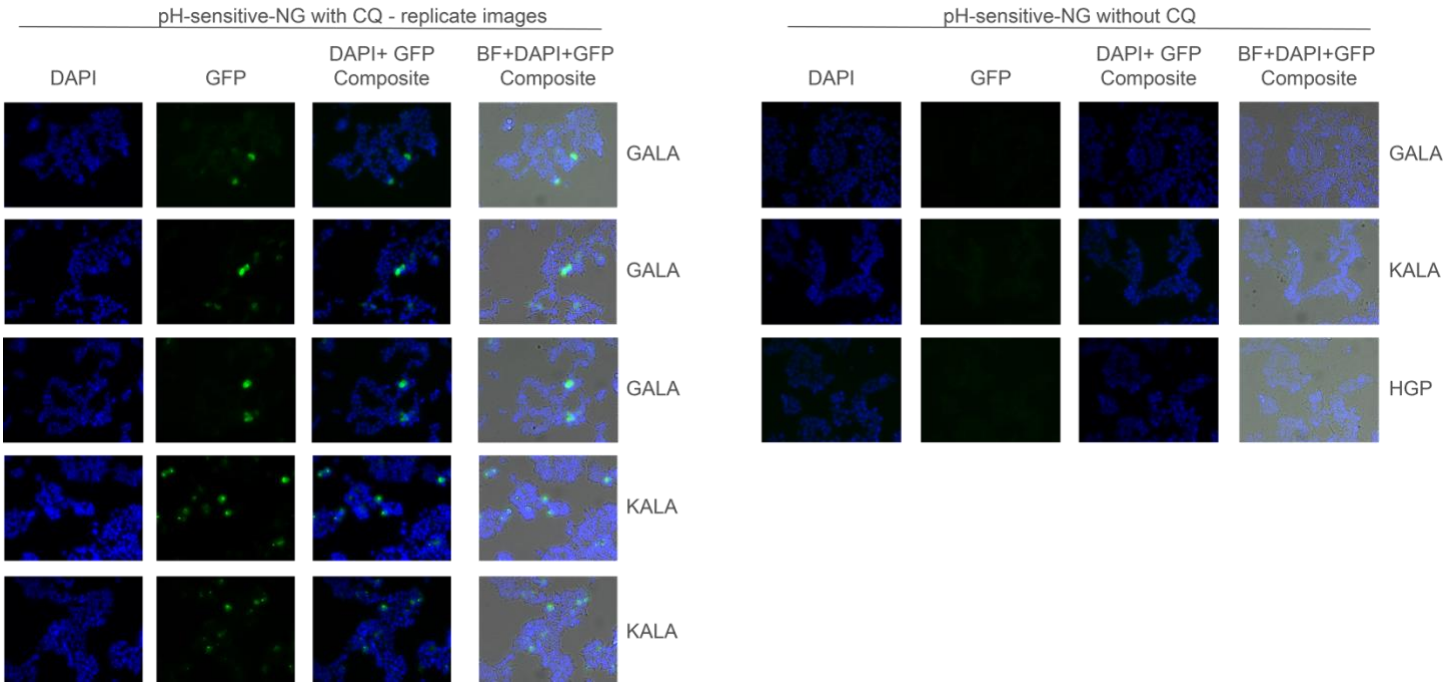


	Xlinked	Functionalized	Size
KALA	17%	11%	12 nm
GALA	17%	15%	18 nm
HGP	17%	14%	13 nm

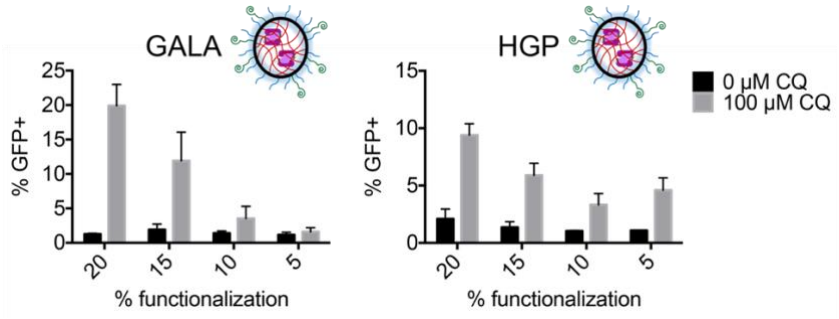
3.X Figure S10. Higher degrees of KALA functionalization increased cellular debris. When HEKs1-10 cells were treated with KALA-NG with high degrees of KALA functionalization, significant cellular debris was observed. Top row: Cells were always gated for the live population, using FSC vs. SS. Bottom row: The live population selected was confirmed using viability dye, 7-AAD.



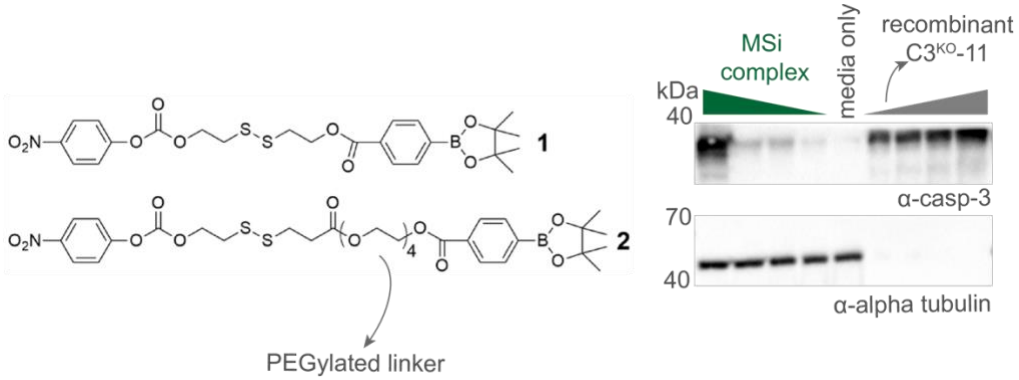
3.XI Figure S11. pH-responsive PEG-PDS Functionalized NG imaging in HEKs1-10. HEKs1-10 cells (seeded in glass-bottom imaging dishes) were incubated with pH-responsive PEG-PDS functionalized NG, in the absence or presence of CQ. Cells were incubated with NG for 24 hours followed by nucleus staining (NucBlue™) and subsequent imaging using 405 nm and 488 nm laser lines.



3.XII Figure S12. Dependency on degree of pH-responsive peptide functionalization. The resultant GFP fluorescence varied for PEG-PDS NG with different extents of GALA- and HGP-functionalization (in the presence and absence of CQ).

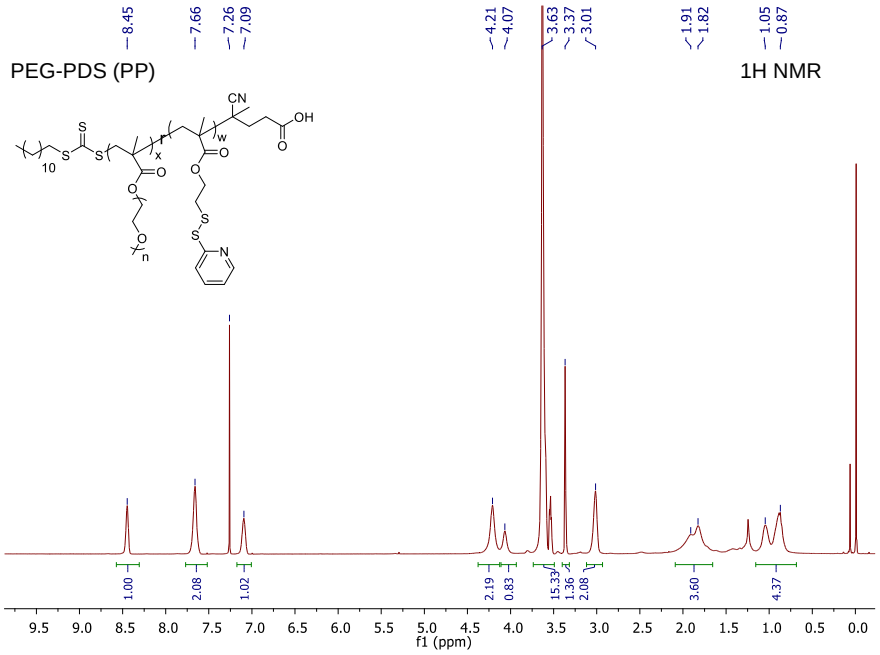


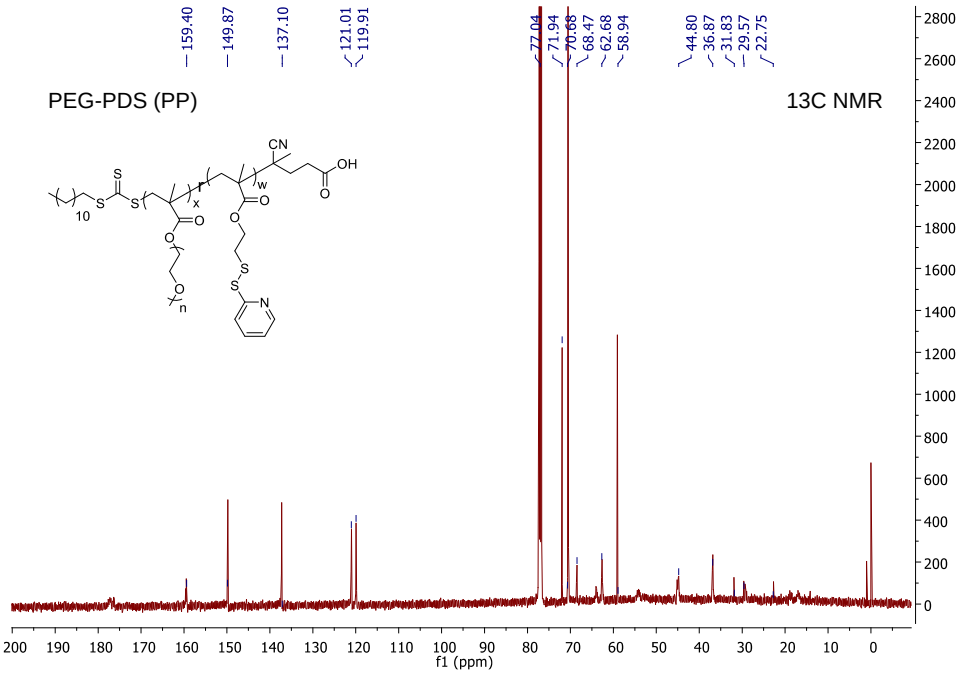
3.XIII Figure S13. MSi linkers used and delivery to MCF-7 cells. MSi linker structures and MSi mediated $C3^{KO}$-11 delivery to MCF7 cells, visualized by western blot.

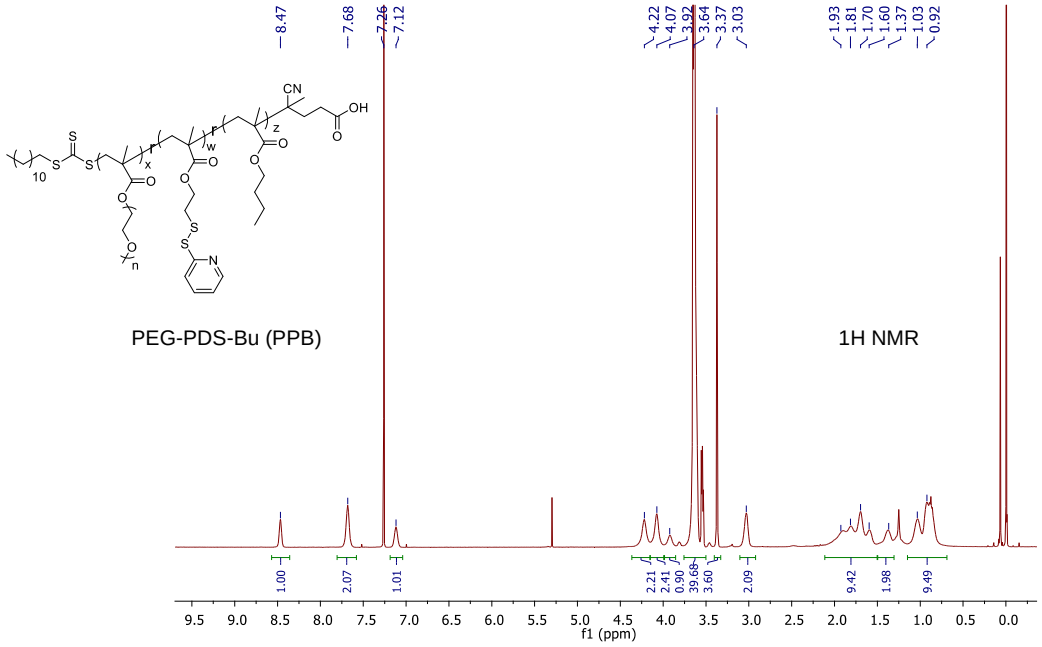


4. NMR characterization

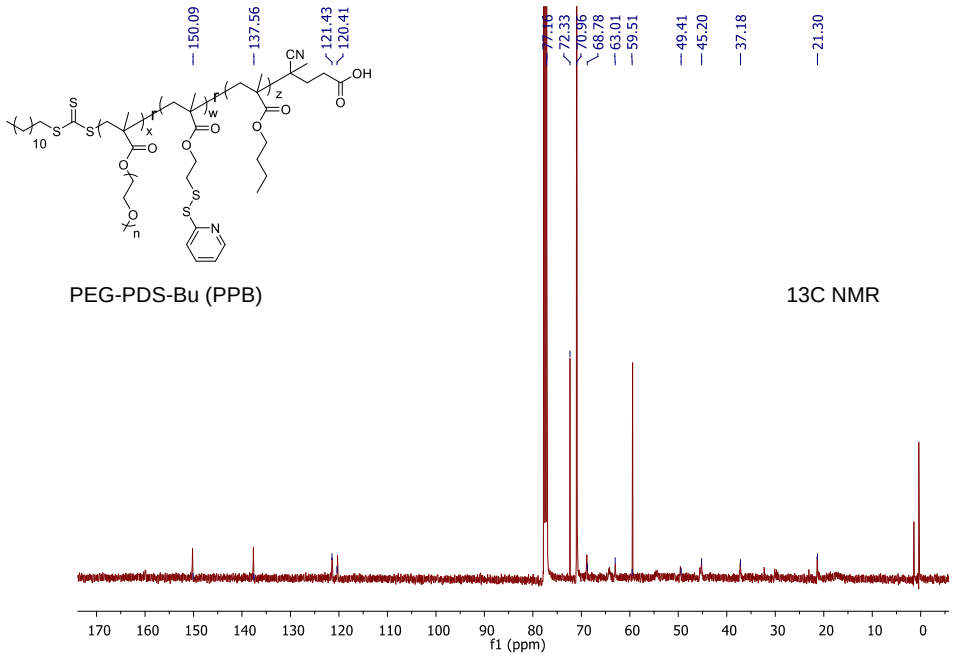
4.I Random copolymers – 1H, 13C NMR



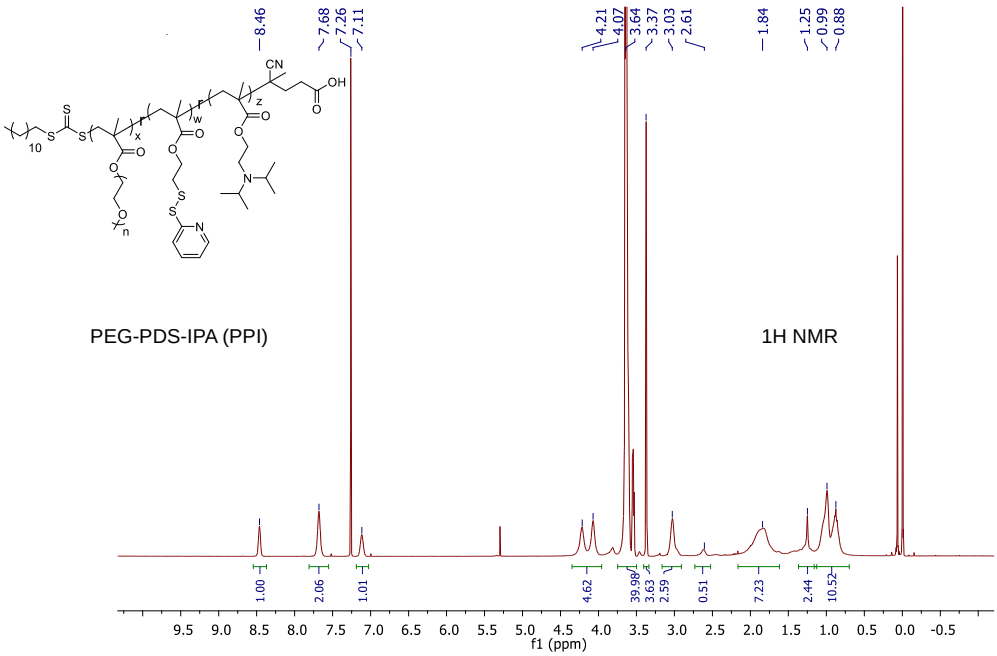




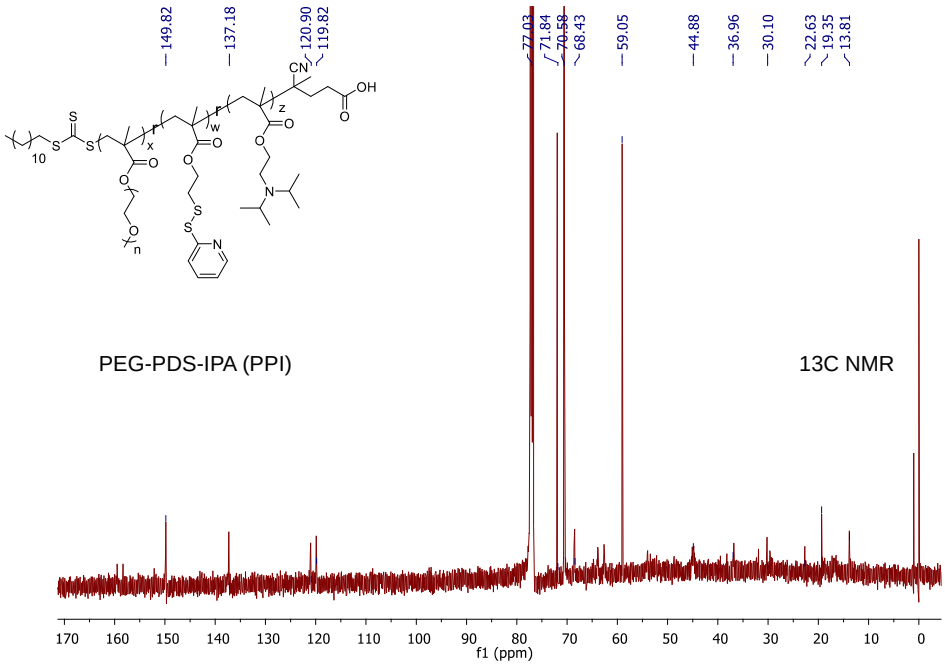
PEG-PDS-Bu (PPB) 1H NMR



PEG-PDS-Bu (PPB) 13C NMR

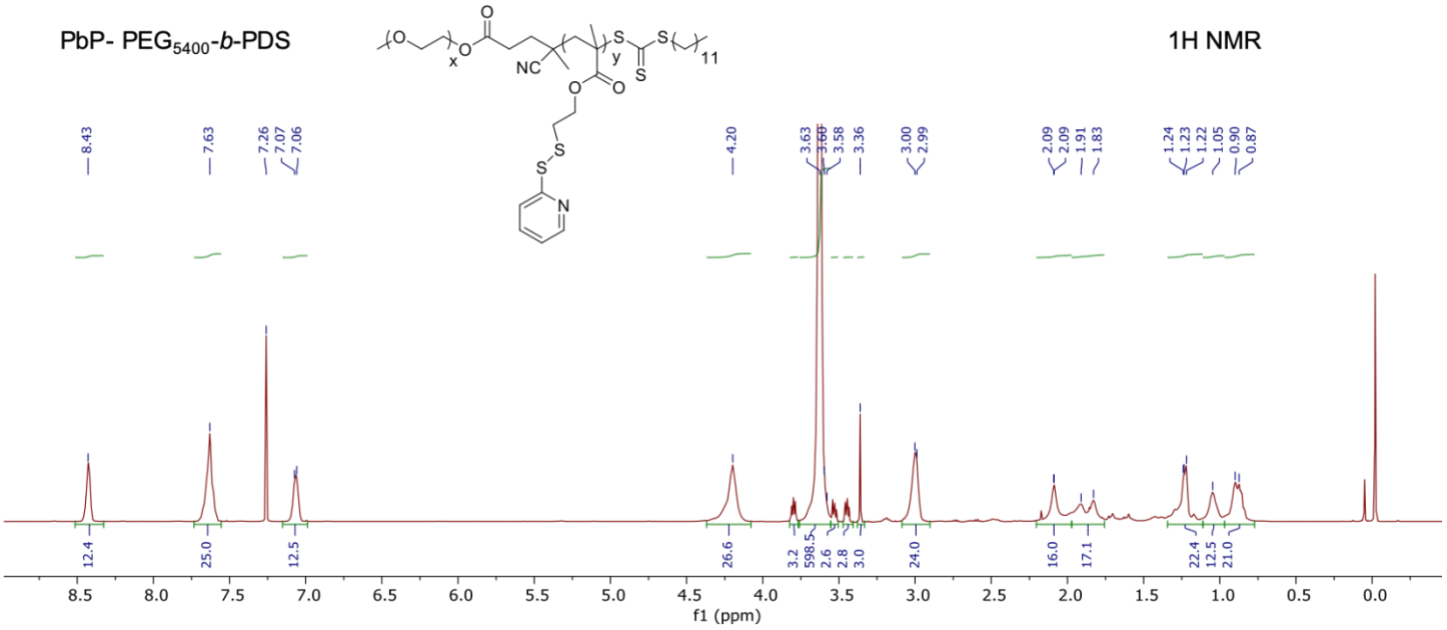


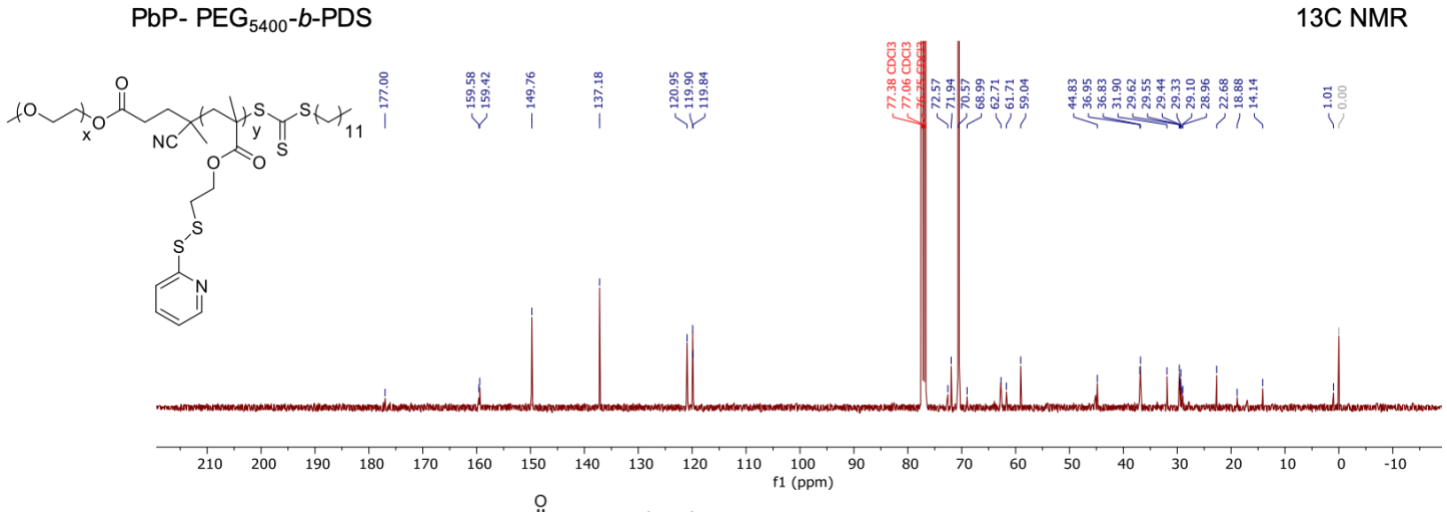
PEG-PDS-IPA (PPI) 1H NMR

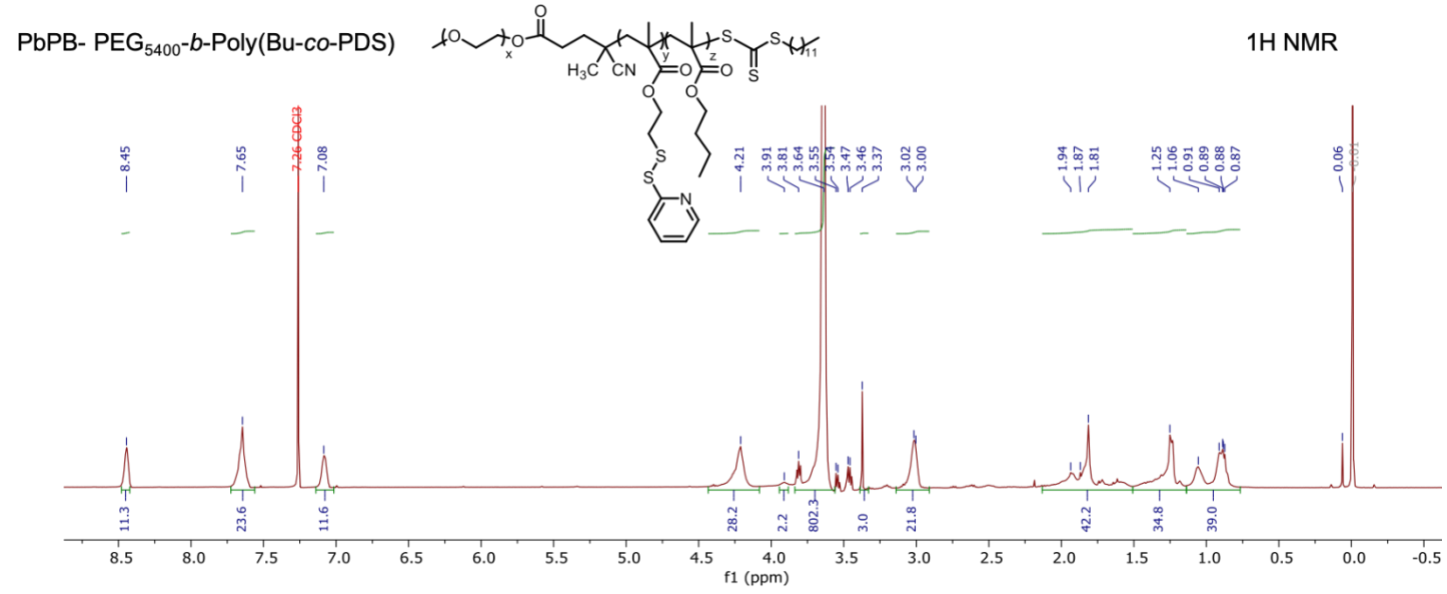


PEG-PDS-IPA (PPI) 13C NMR

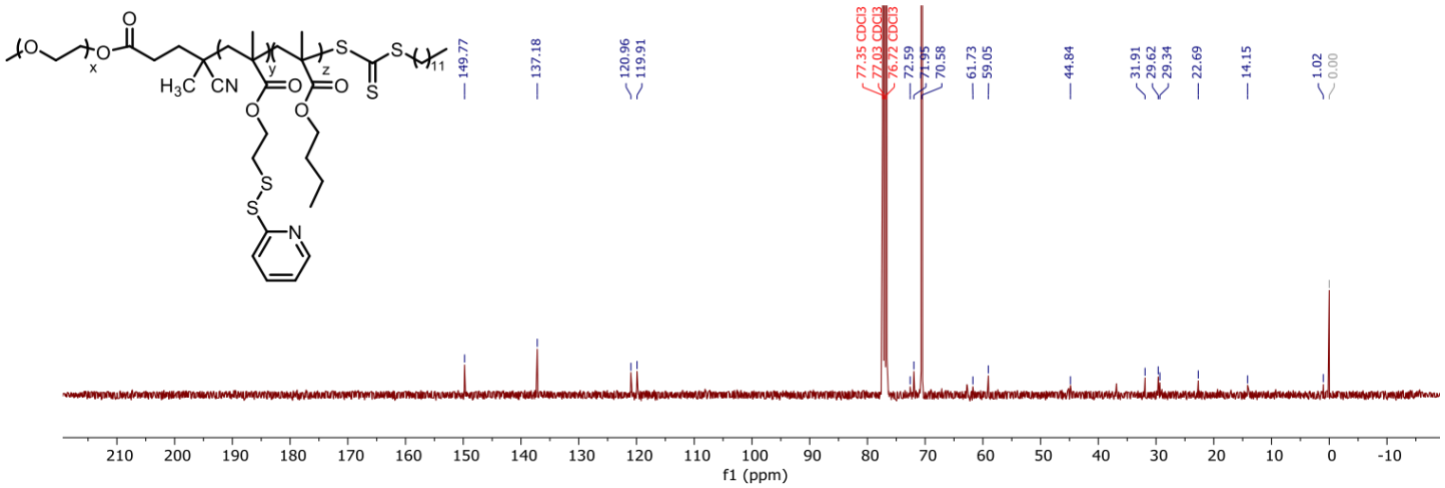
4.II Block copolymers – 1H, 13C NMR







PbPB- PEG$_{5400}$-*b*-Poly(Bu-*co*-PDS) 13C NMR



4. Supplemental References:

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