Electronic Supplementary Material (ESI) for ChemComm.

Supporting Information

A pH-sensitive eosin-block copolymer for intracellular protein delivery

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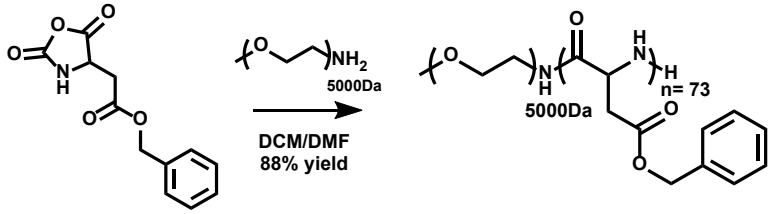
Experimental section

All commercially available compounds were purchased from Sigma Aldrich and all solvents were purchased dry from Sigma Aldrich with a Sure/Seal system. ^{1}H NMR spectra were recorded on a Bruker Avance 400 spectrometer at 400 MHz.

Synthesis of PEG-pAsp(Bz)



PEG-pAsp(Bz)

To a solution of benzyl 2-(2,5-dioxooxazolidin-4-yl)acetate (1.0 g, 4 mmol, 100eq) in 10:1 of DCM:DMF (4.5mL) was added a solution of MeO-PEG5000-NH2 (0.2 g, 0.04 mmol, 1eq) in 10:1 of DCM:DMF (7mL). The solution was heated to 40^{o}C and allowed to stir for 48 hours. The mixture was then cooled to room temperature and added to cold ethyl ether (125 mL). The resulting precipitate was filtered and washed with additional ethyl ether (100mL). This yielded the product PEG-pAsp(Bz) as a white solid (898.7mg, 88% yield). NMR analysis indicated that the degree of polymerization was approximately 73. The ^{1}H NMR spectrum is in the Analytical Data section.

Synthesis of PEG-pAsp-ketal-amine

To 2,2-Bis(aminoethoxy)propane (827 mg, 5.1 mmol, 3000eq) was added dropwise over 10 mins a solution of PEG-pAsp(Bz) (29.4 mg, 1.7 umol, 1eq) in NMP (1 mL). The solution was allowed to stir at room temperature for 16 hours. The mixture was added to cold ethyl ether (5 mL). The resulting precipitate was filtered and washed 3 times with cold ethyl ether (5 mL). This yielded the product PEG-pAsp-ketal-amine as a white solid (29.7 mg, 85% yield). The ^{1}H NMR spectrum is in the Analytical Data section

Synthesis of PEG-pEosin

PEG-pAsp(ketal) (10 mg, 0.476 µmol) was dissolved in 0.5 mL of anhydrous DMF. To this solution were added DIPEA (9.23 mg, 13.2 µL, 71.4 µmol) and Eosin 5-isothiocyanate (49.8 mg, 70.6 µmol, dissolved in 1 mL anhyd. DMF), both in 2.5-fold molar excess per free amine of PEG-pAsp(ketal). The reaction mixture was stirred at room temperature for 120 min. The reaction solution was added dropwise to 40 mL of cold n-hexane/ether 50/50. The red precipitate was collected and then redissolved in 0.2 mL of DMF, and precipitated with cold n-hexane/ether 50/50 3 times, and dried under vacuum. The red solid was dissolved in 2 mL 0.1% EtN_3 in water and further purified using a Zeba™ Spin Desalting Column, 7K MWCO, 10 mL to afford PEG-pEosin as a red powder after lyophilization. The yield was 22.6 mg, 76%. PEG-pEosin was readily soluble in water at a concentration of 10 mg/mL (161 µM). The ^{1}H NMR spectrum is in the Analytical Data section.

Preparation of protein formulations with PEG-pEosin

PEG-pEosin and proteins were diluted separately in equal volumes of phosphate-buffered saline (PBS) and mixed at indicated ratios by rapid pipetting. The formulations were incubated for 5 min at room temperature.

Native polyacrylamide gel electrophoresis

Bovine serum albumin (BSA) formulations with PEG-pEosin were prepared at indicated ratios. In the case of binding studies, the formulations were loaded immediately into the gels. In the case of release studies, the formulations were incubated for 4 hours at room temperature in PBS at the indicated pH before loading into the gels. BSA formulations contained 1.5 µg protein in a 15 µL volume and were pipetted into the well after mixing with 5 µL of loading buffer (prepared from 4 mL of glycerol, 6 mL running buffer and 0.02 g of bromophenol blue). Native 4–20% Mini-PROTEAN® TGX™ Precast Protein Gels (Biorad) were used. The running buffer contained 3.0 g of Tris base and 14.4 g of glycine in 1000 mL of H_2O. The pH of the buffer was 8.3. Native PAGE was performed at 70 V until samples were stacked and subsequently at 140 V. Gels were stained with Bio-Safe™ Coomassie Stain (Biorad).

Particle size and zeta potential

For dynamic light scattering (DLS) measurements the protein formulations were measured in a folded capillary cell (DTS 1070) using a Zetasizer Nano ZS with backscatter detection (Malvern Instruments). Proteins were mixed with

a 6-fold molar excess of PEG-pEosin in a total volume of 50 µL containing 10 µg protein. Samples were measured in triplicates. For zeta potential measurements, the samples were diluted with 750 µL PBS. The influence of acid hydrolysis on the particle distribution was studied by measuring DLS after 10 min incubation in PBS at pH 5.5.

Hemolysis assay

Fresh rabbit blood (900 µL) was washed 3 times with PBS. The erythrocyte suspension was centrifuged at 1500 RCF for 0.5 min and the pellet was resuspended in 2 mL PBS. The suspension was diluted 1:20 in PBS at pH 7.4, 6.8 and 5.5. Volumes of 400 µL of erythrocyte suspension and 400 µL of LLO solutions (with or without PEG-pEosin, previously formulated with PBS at the respective pH to achieve a 0.5 µg/mL final concentration of LLO) were incubated for 2 hours at 37°C. At indicated time points, aliquots of 100 µL were centrifuged and 60 µL of the supernatant was analyzed for hemoglobin release at 405 nm wavelength using a TECAN infinity 200 microplate reader. Samples were analyzed in triplicates. PBS at the respective pH was used as a negative control (0% lysis) and 1% solutions of Triton-X in PBS was used as a positive control (100% lysis).

Cell viability assay

Cell viability of HeLa cells was analyzed after incubation with free and PEG-pEosin-formulated LLO at 2.5 µg/mL concentration at pH 7.4 and 6.8 for 4 hours in serum-free media and 24 hours at different concentrations in serum-containing media. Cell viability of 3T3 Ai9 cells was analyzed after 24 hours incubation with Cre recombinase co-formulations with 8 and 12 µg/mL concentrations of LLO and 6 molar equivalents of PEG-pEosin in comparison to controls with Cre only, PEG-pEosin and Cre + free LLO in serum-containing media. The cells were washed with PBS and incubated with 100 µL fresh medium containing resazurin (10 µL 0.1mg/mL in PBS) for 3 h. The cell viability was then determined by measuring the fluorescence intensity of each well (Ex = 550 nm, Em = 595 nm), using a TECAN infinity 200 microplate reader. Untreated cells and fresh medium were used as controls for 100% and 0% cell proliferation, respectively.

Cre recombinase assay

Gene editing efficiency of Cre recombinase co-formulations was analyzed in 3T3 Ai9 cells, using 8 µg/mL (143 nM) and 12 µg/mL (214 nM) concentrations of LLO, 3.8 µg/mL (100 nM) Cre and 6 molar equivalents of PEG-pEosin (1.46 µM and 1.88 µM respectively). The controls were composed of Cre only, PEG-pEosin, and Cre + free LLO. The formulations were incubated with 3T3 Ai9 cells for 48 hrs at 37 °C in a humidified incubator containing 5% CO_2. The 3T3 Ai9 cells were detached with Accutase (Invitrogen) and the TdTomato signal was analysed with a Attune NxT flow cytometer (Invitrogen). The gene editing efficiency was determined by identifying the percentage of TdTomato positive cells.

Stereotaxic injection

Male and female Ai9 (Cre-dependent tdTomato reporter) mice (Jackson Laboratory, stock #007909) aged 2–5 months were used in this study. Mice were anaesthetized using isoflurane (4% for induction and 2% for maintaining) and injected with 2 µL of either CRE+LLO+PEG-pEosin (final concentration per injection; PEG-pEosin: 53.5 µM, LLO: 6.1 µM, Cre: 2.8 µM) or saline into the striatum (AP: 0.50 mm, ML: +1.87 mm, DV: -3.47 mm; 0.4 µl/min) using a Hamilton Neuros syringe. After the infusion, the syringe was slowly removed and the scalp sutured. A period of 20 days was given before the perfusion and histology procedures. The use and care of animals in this study followed the guidelines of the UTHSCSA and UC Berkeley Institutional Animal Care and Use Committee.

Histology

20 days after stereotaxic injection, the mice were anaesthetized by isoflurane and were perfused with ice-cold PBS followed by 4% paraformaldehyde (PFA) in PBS. The brains were post-fixed for 4 h in 4% PFA, washed once with PBS, and then transferred to cryoprotective 30% sucrose in PBS at 4°C. After cryoprotection, the brains were embedded in O.C.T. compound, frozen, and then stored at −80°C until cryosectioning. Mouse brain sections were obtained by cryostat (CM3050S; Leica Microsystems). Slices with striatum were cryosectioned on the coronal plane at 20 µm, mounted on glass slides, and stored at 4°C. The sections to be immunostained were washed three times in PBS and than incubated with DAPI diluted in PBS (1:1500 of 5 mg/mL stock solution) for 10 min. After the incubation, the sections were washed three times in PBS and mounted with Prolong Gold Antifade Reagent and imaged using a Zeiss confocal microscope with 20x objective.

Supplement figures and tables

Supplement figures

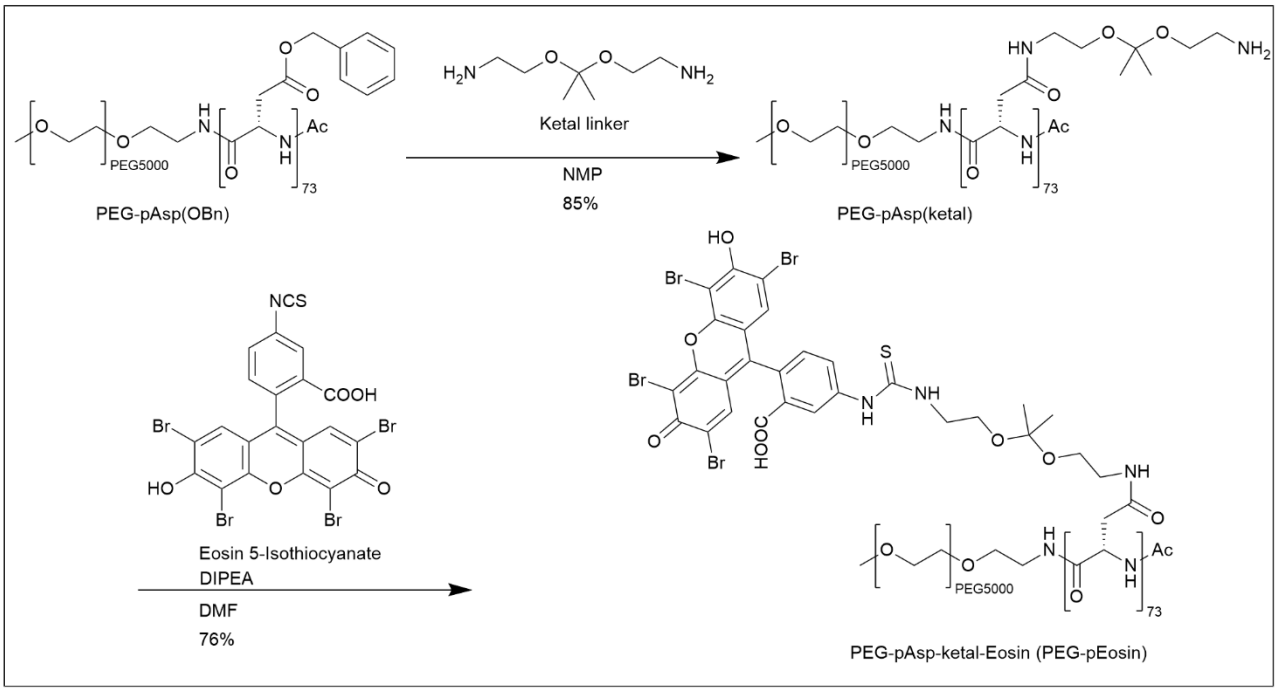


Fig. S1 Synthesis of PEG-pEosin. PEG-pEosin is comosed of a block polymer that consists of a PEG chain and poly(aspartic acid) block, the protein binding dye eosin is bound to the poly(aspartic acid) block via a pH-sensitive ketal linker. A two-step synthetic route was used to generate PEG-pEosin. Briefly, PEG-pAsp(OBn) polymer was reacted with a diamine ketal linker to generate PEG-pAsp(ketal), the PEG-pEosin polymer was synthesized by reacting this interemdiate with eosin 5-isothiocyanate.

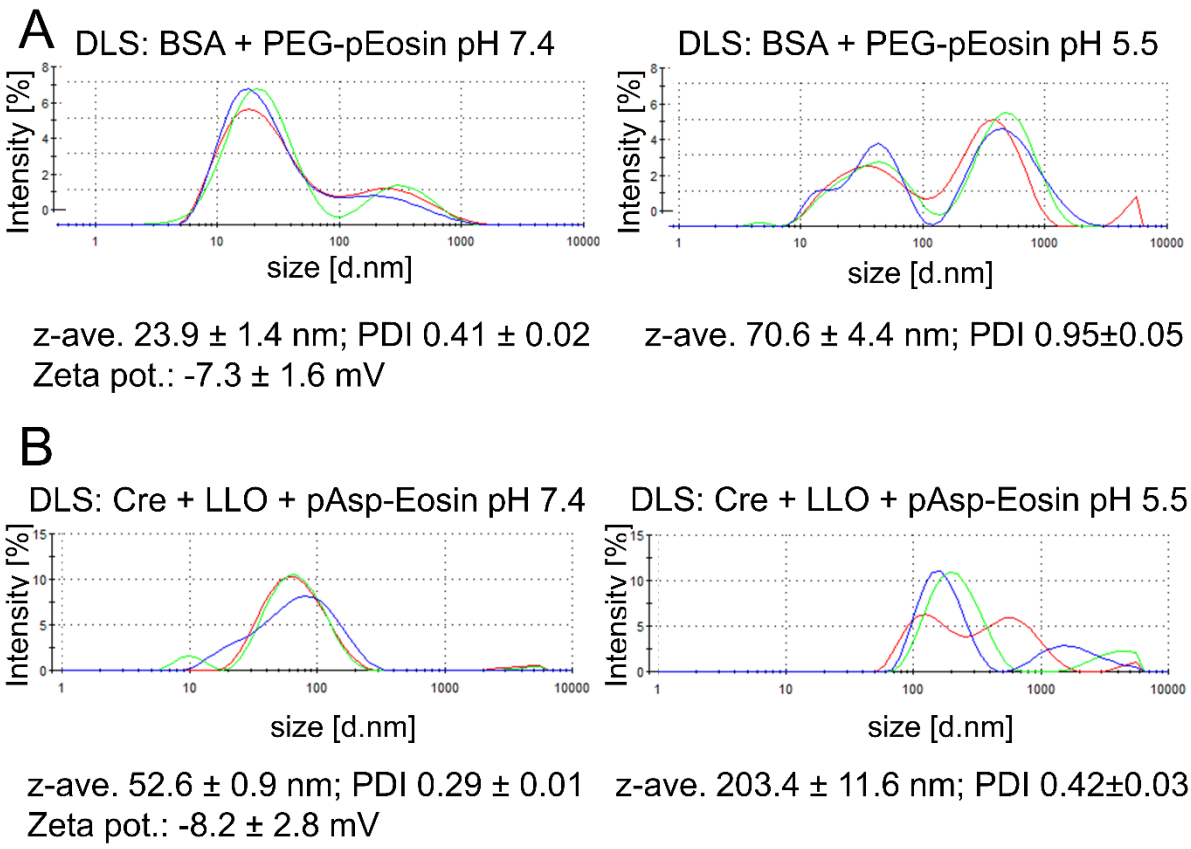


Fig. S2 Dynamic light scattering (DLS) measurements of PEG-pEosin formulations with A) BSA and B) Cre and LLO at neutral pH (left) and under acidic conditions (right).

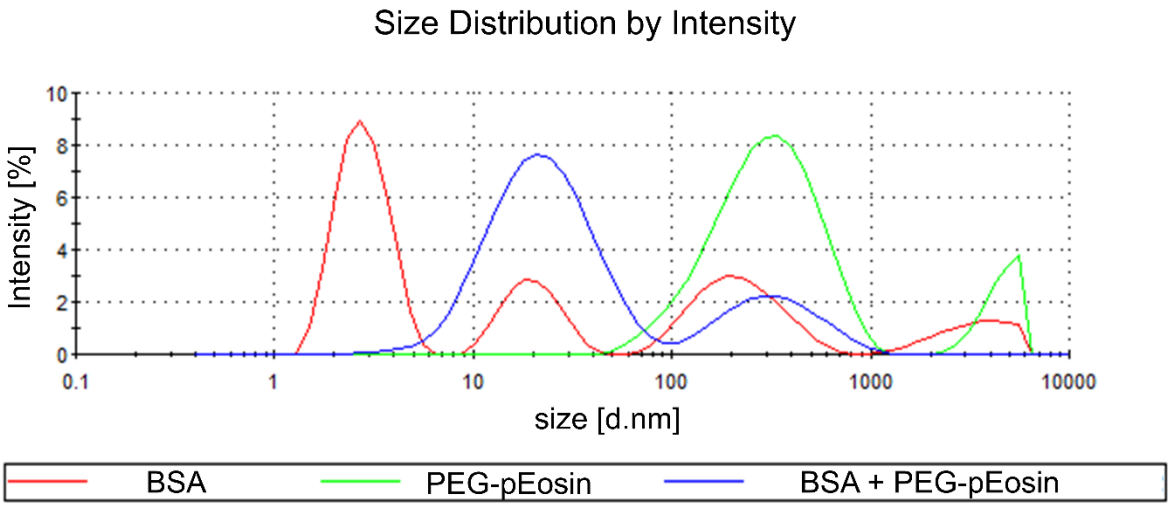


Fig. S3 Overlay of DLS measurements of free BSA, PEG-pEosin alone, and the BSA + PEG-pEosin formulation.

Supplement tables

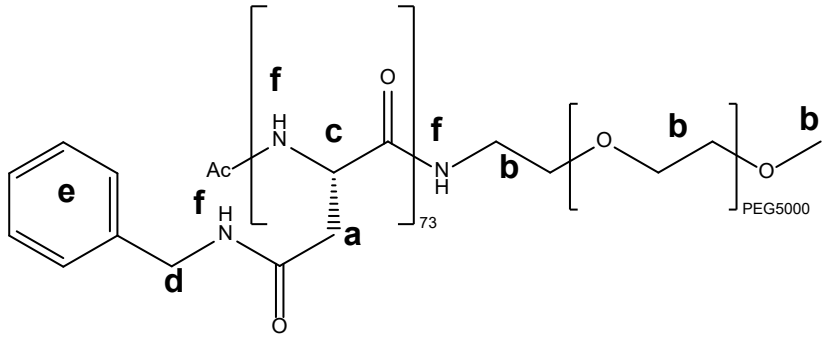
Sample	z-average [nm]	PDI	Zeta potential [mV]
BSA	12.6 ± 1.6	0.44 ± 0.02	- 6.7 ± 2.3
PEG-pEosin	335 ± 63	0.55 ± 0.21	- 13.9 ± 5.4
BSA+PEG-pEosin	23.9 ± 1.4	0.41 ± 0.02	- 7.3 ± 1.6
Cre+LLO+PEG-pEosin	52.6 ± 0.9	0.29 ± 0.01	- 8.2 ± 2.8

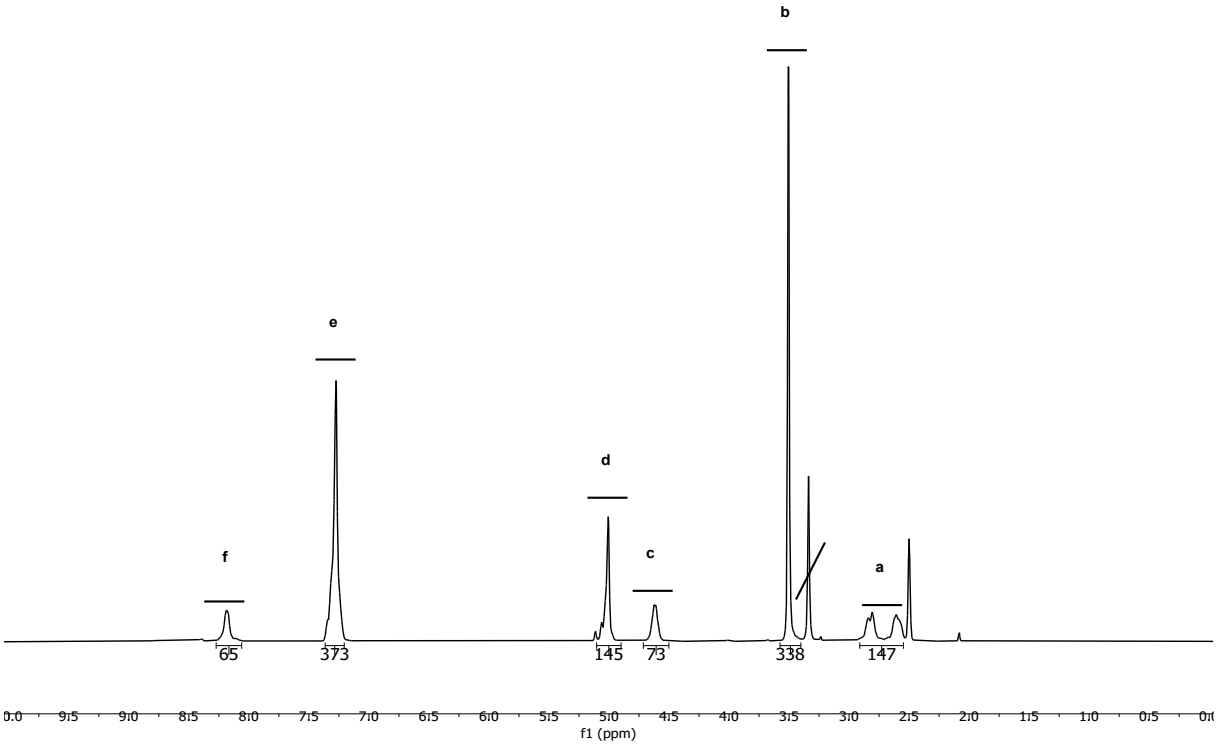
Table. S1 Summary table of DLS measurements of BSA and PEG-pEosin alone as well as formulations with PEG-pEosin and BSA or Cre and LLO at neutral pH.

Analytical data

^{1}H Proton NMR spectra

PEG-pAsp(Bz)



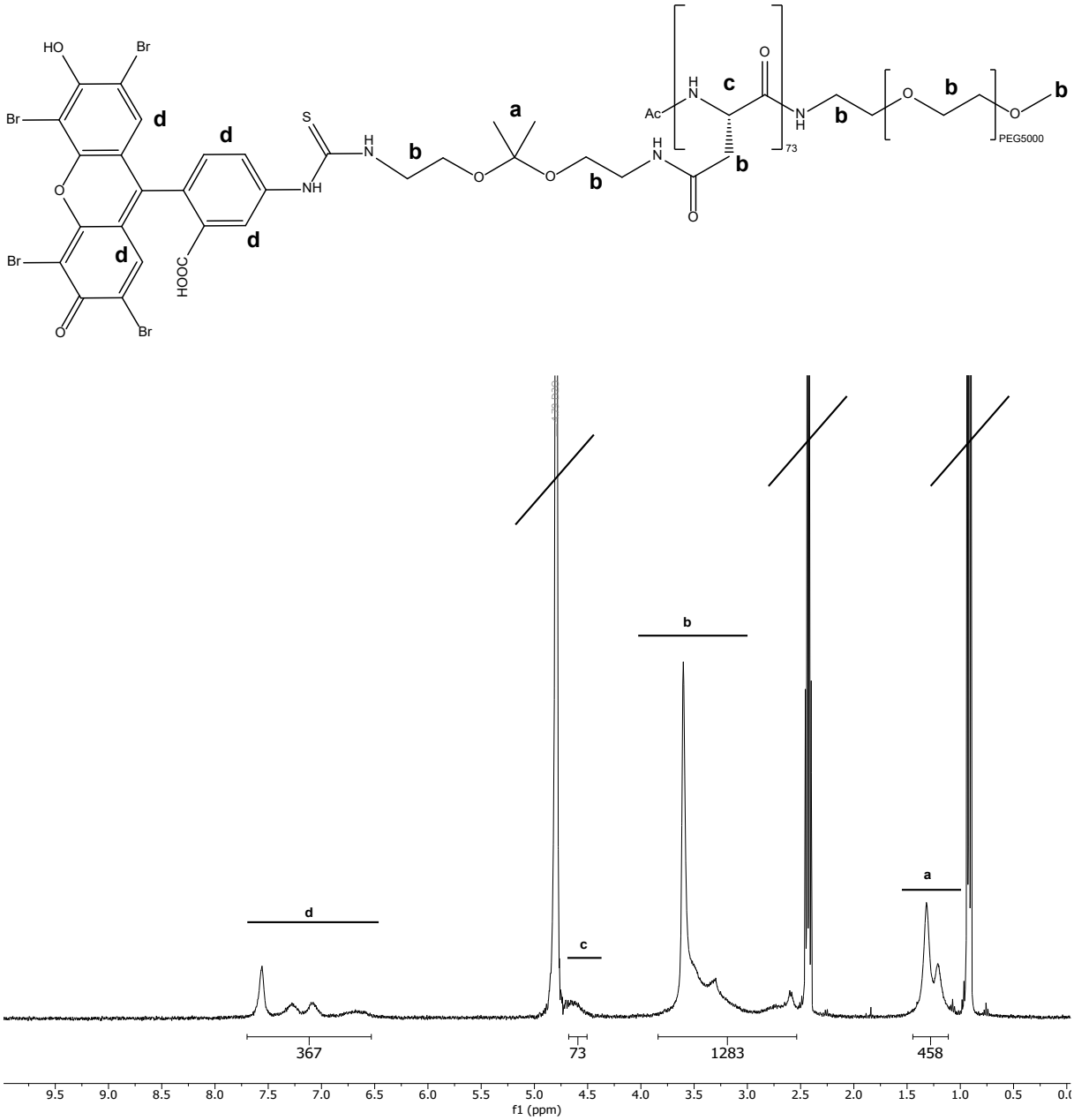


1H NMR (400 MHz, DMSO) δ (ppm) = 2.55 – 2.91 (m, 147H, βH Asp), 3.40 – 3.58 (s, 338H, O-CH2-CH2-O and OCH3 PEG), 4.50 – 4.71 (m, 73H, αH Asp), 4.90 – 5.10 (m, 145H, CH_2 benzyl groups), 7.21 – 7.36 (m, 373H, -CH- benzyl groups), 8.06 – 8.27 (m, 65H, -NH)

PEG-pAsp-ketal-amine

^{1}H NMR (400 MHz, DMSO:Deuterium oxide 4:1) δ (ppm) = 1.10 - 1.40 (s, 441H, -CH_3 ketal linker), 2.51 - 2.80 (s, 195H, βH Asp), 2.94 – 3.54 (m, 853H, O-CH_2-CH_2-O and OCH_3 PEG, N-CH_2-CH_2-O ketal linker), 4.35 - 4.63 (m, 73H, αH of Asp)

PEG-pEosin



^{1}H NMR (400 MHz, Deuterium oxide, 0.1% Triethylamine) δ (ppm) = 1.13-1.39 (458 H, -CH_3 ketal linker), 2.88-3.84 (1387 H, O-CH_2-CH_2-O and OCH_3 PEG, βH Asp, N-CH_2-CH_2-O ketal linker), 4.51- 4.68 (79 H, αH of Asp), 6.53-7.70 (397 H, -CH- of eosin).