Electronic Supplementary Information (ESI)

Effect of Polymer Topology on non-covalent Polymer-Protein Complexation: Miktoarm versus Linear mPEG-poly(glutamic acid) Copolymers

Alejandro Nieto-Orellana,[a] Marco Di Antonio,[b] Claudia Conte, [a] Franco H. Falcone,[a] Cynthia Bosquillon,[a] Nick Childerhouse,[c] Giuseppe Mantovani*[a] and Snow Stolnik*[a]

Table of Contents

1. Materials

L-Glutamic acid γ-benzyl ester (≥99.0%), allyl bromide (97%), cysteamine hydrochloride (≥98%), triethylamine (≥99.0%), sodium phosphate dibasic (99.9%), sodium phosphate monobasic dehydrate (≥99.0%), phosphoric acid*, Micrococcus lisodeikticus* lyophilised cells, poly(allyl amine) solution (M_w ~17 kDa, 20 wt. % in H_2O), tris-acetate-EDTA buffer, agarose, acetonitrile (ACN, anhydrous, 99.8%), methoxypolyethylene glycol amine 2,000, dichloromethane (DCM, anhydrous, ≥99.8%), *N,N*-Dimethylformamide (DMF, anhydrous, 99.8%), tetrahydrofuran (THF, anhydrous, ≥99.9%), deuterated chloroform (99.8 atom % D), deuterated dimethyl sulfoxide (99.9 atom % D), deuterium oxide (99.9 atom % D) and deuterated methanol (99.8 atom % D) were purchased from Sigma Aldrich. Methyl 3,4,5-trihydroxybenzoate (98.0%), potassium carbonate anhydrous (≥99.0%), sodium hydroxide (≥97.0%), oxalyl chloride (98.0%), 2,2-dimethoxy-2-phenylacetophenone (99.0%), hydrochloric acid, ethidium bromide, magnesium sulphate dried, toluene, methanol, diethyl ether anhydrous, propan-2-

ol, petroleum ether and ethyl acetate were supplied by Fisher Scientific. Triphosgene 98.0% was purchased from Alfa Aesar. Lysozyme (from chicken egg white) molecular biology grade was supplied by AppliChem. All the chemicals were used as received without further purification. Anhydrous solvents were used as received and stored under dry and inert atmosphere. Thin layer chromatography (TLC) was carried out on pre-coated TLC sheets ALUGRAM®SIL G/UV254 purchased from Macherey-Nagel. TLCs were visualized by exposure to UV light (254nm) followed by staining with $KMnO_4$.

2. Instrumentation

2.1. **Characterisation of polymers and intermediates.** ^{1}H and ^{13}C NMR spectra were recorded at room temperature on a Bruker DPX 400 MHz using deuterated solvents ($CDCl_3$, CD_3OD or D_2O). All chemical shifts are reported in parts per million (ppm) and using residual solvent as an internal standard ($CDCl_3$: δH 7.26, δC 77.16; D_2O: δH 4.79; CD_3OD: δH 3.31). The mass spectrometric analyses were carried out using a Micromass LCT KC453 ESI-TOF spectrometer and an Ultraflex III MALDI-TOF spectrometer with N_2 laser of 337nm and pulses of 3ns. DCTB was used as matrix. FT-IR analyses were performed on a Nicolet IR200 FT-IR spectrometer (Thermo Fischer Scientific).

2.2. **Size Exclusion Chromatography (SEC).** Aqueous SEC was performed on a Shimadzu UPLC system fitted with a differential refractive index detector. The mobile phase was aqueous 0.10 M $NaNO_3$, 0.02 (w/v) NaN_3, pH 10.0 at 35ºC and a flow rate of 1.0 $mL{\cdot}min^{-1}$. The instrument was fitted with a Polymer Labs aquagel-OH guard column (50 x 7.5 mm, 8 µm) followed by three PL aquagel-OH columns (30, 40 and 50; 300 x 7.5 mm, 8 µm). Column calibration was achieved using PEG/ (PEO) narrow standards with Mp in the 0.200 – 130 kDa range. Molecular weights and dispersity values were calculated using Shimadzu LabSolutions software with SEC analysis add-on.

2.3. **Transmission electron microscopy (TEM).** TEM analysis was performed using a Tecnai G2 (FEI, Oregon, USA). Images were captured using Megaview III and analysed by Megaview III Soft imaging system. Lysozyme-mPEG$_{2k}$-*lin*-GA$_{30}$ nanocomplexes prepared at r 2.5 were loaded at a protein concentration of 70 µg mL^{-1}. Samples were prepared by placing complexes suspension onto 3.05 mm Nickel grid, Formvar/Carbon supported film, 100 HEX. No staining was used in the sample preparation.

3. Characterisation of copolymers and corresponding precursors.

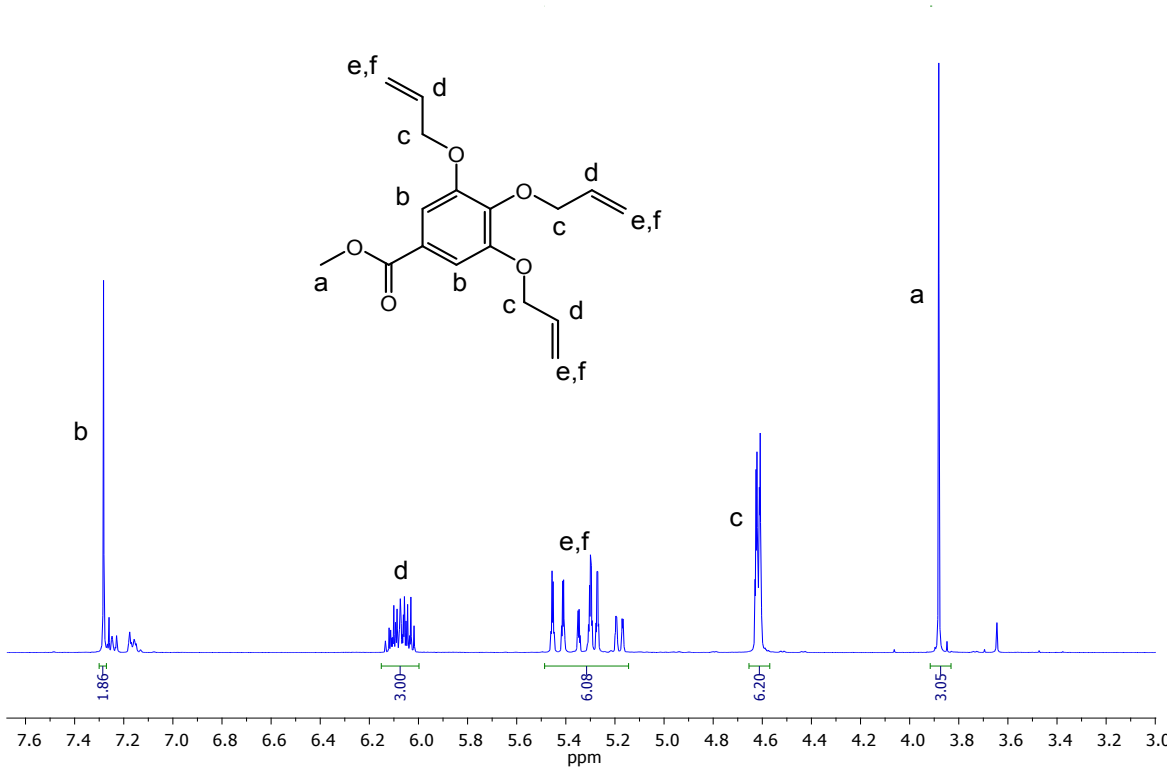

Figure S1. ^{1}H NMR spectrum of 3,4,5-tris(allyloxy)benzoic acid methyl ester **(1)** in $CDCl_3$.

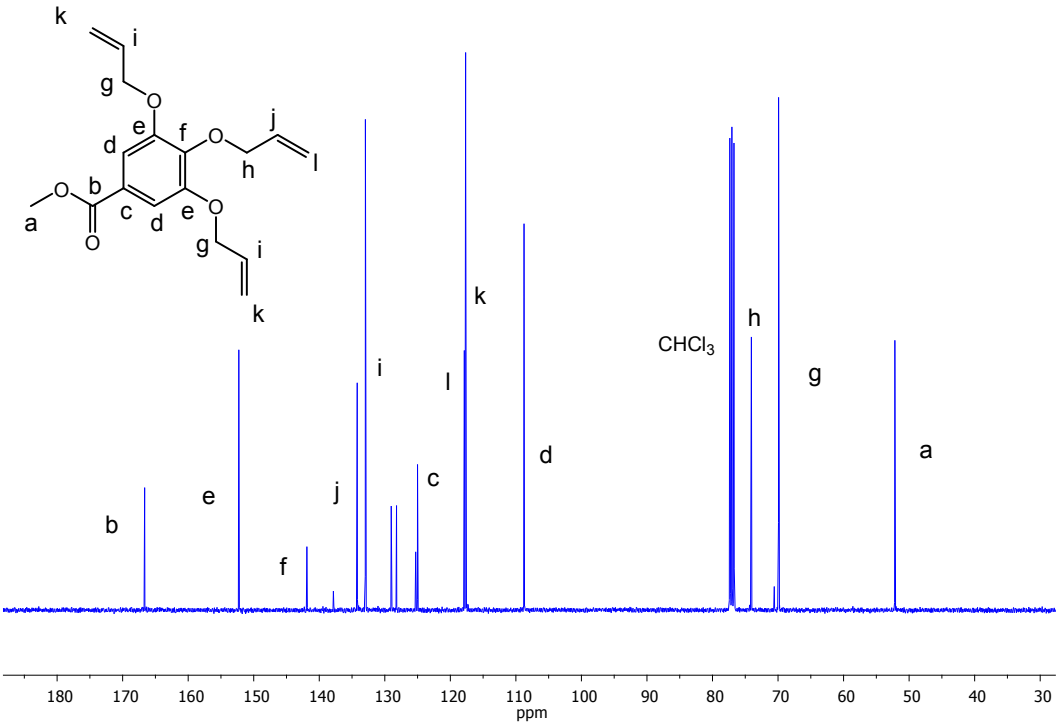

Figure S2. ^{13}C NMR spectrum of 3,4,5-tris(allyloxy)benzoic acid **(1)** methyl ester in $CDCl_3$.

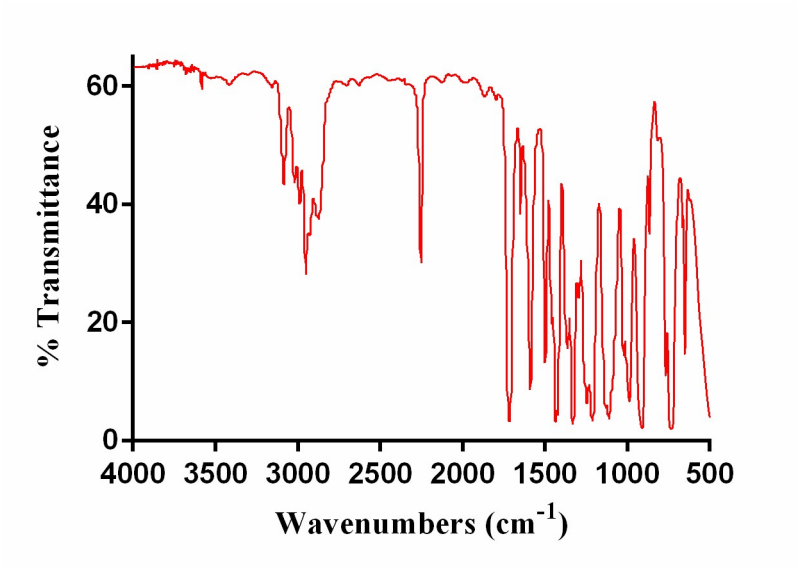

Figure S3. FT-IR spectrum of 3,4,5-tris(allyloxy)benzoic acid methyl ester **(1)**.

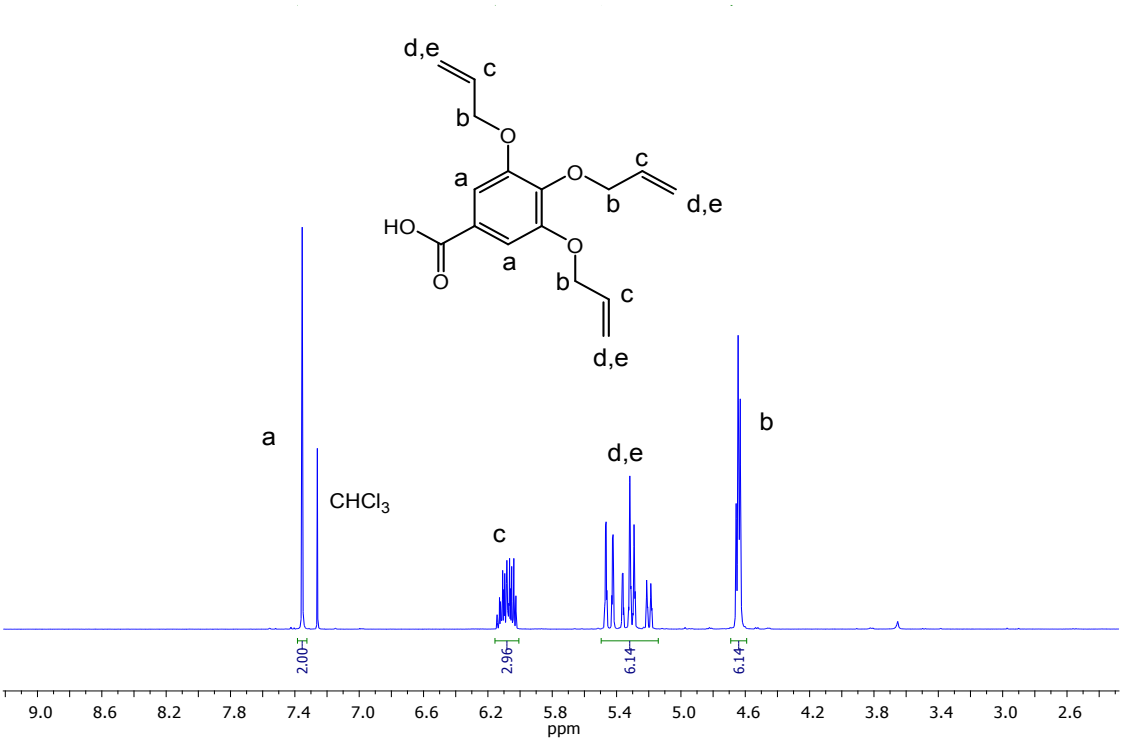

Figure S4. ^{1}H NMR spectrum of 3,4,5-tris(allyloxy)benzoic acid **(2)** in $CDCl_3$.

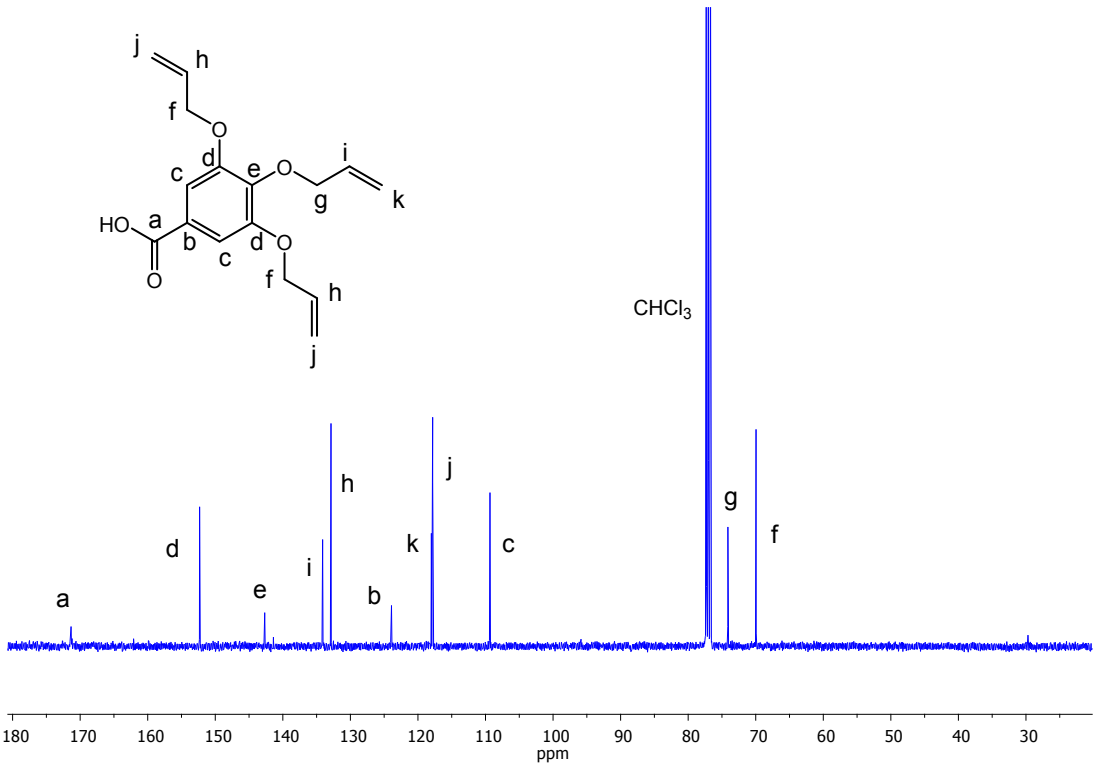

Figure S5. ^{13}C NMR spectrum of 3,4,5-tris(allyloxy)benzoic acid **(2)** in $CDCl_3$.

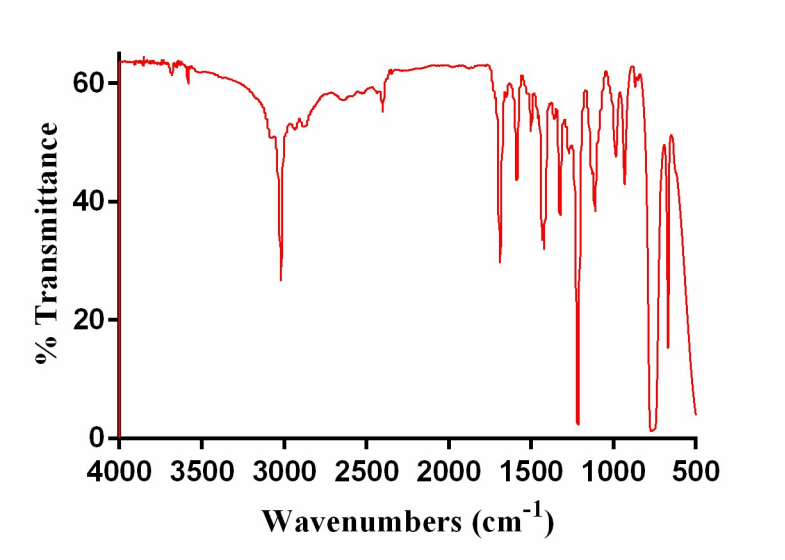

Figure S6. FT-IR spectrum of 3,4,5-tris(allyloxy)benzoic acid **(2)**.

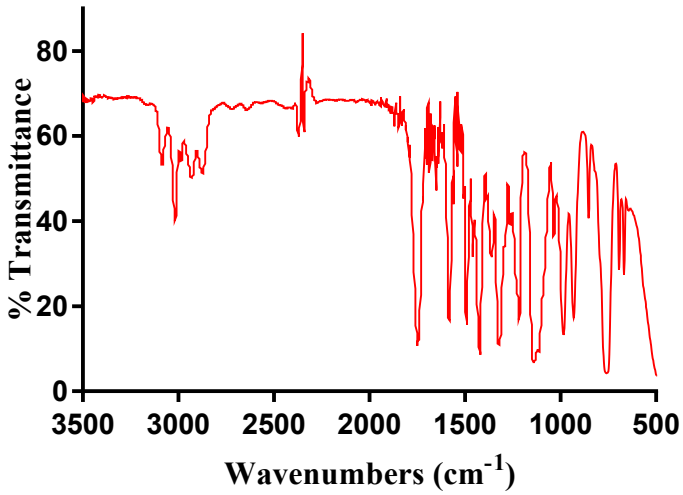

Figure S7. FT-IR spectrum of 3,4,5-tris(allyloxy)benzoyl chloride **(3)**.

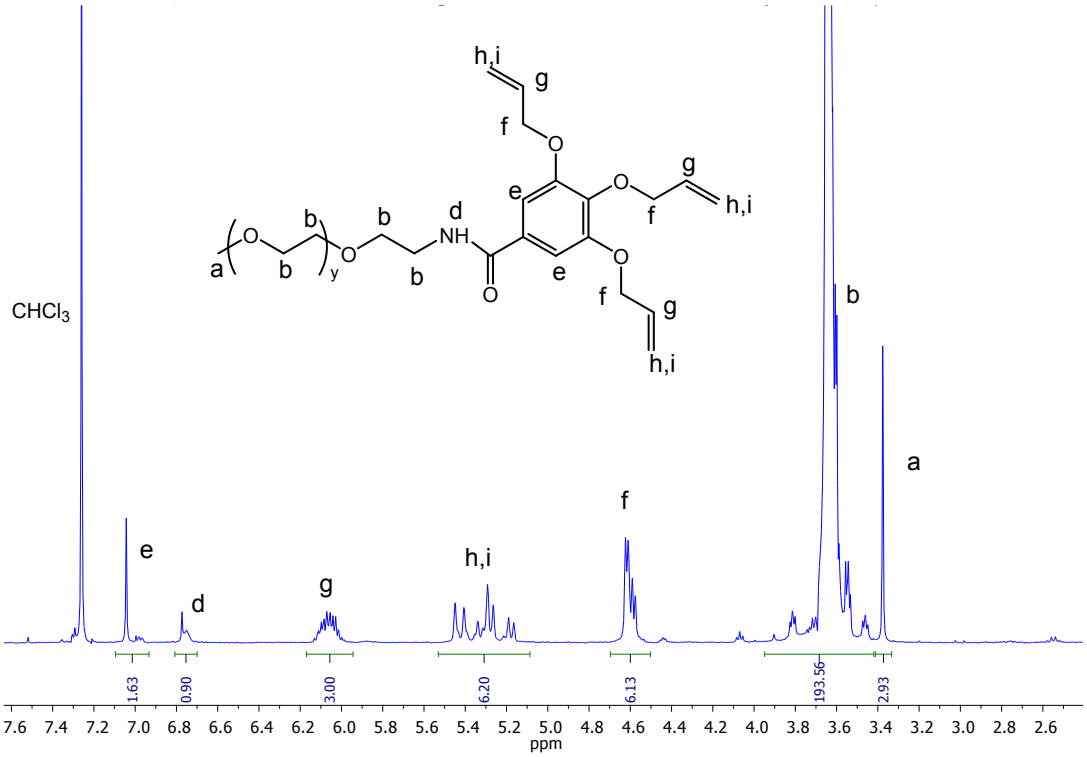

Figure S8. ^{1}H NMR spectrum of $mPEG_{2k}$ triallyl ether **(4)** in $CDCl_3$.

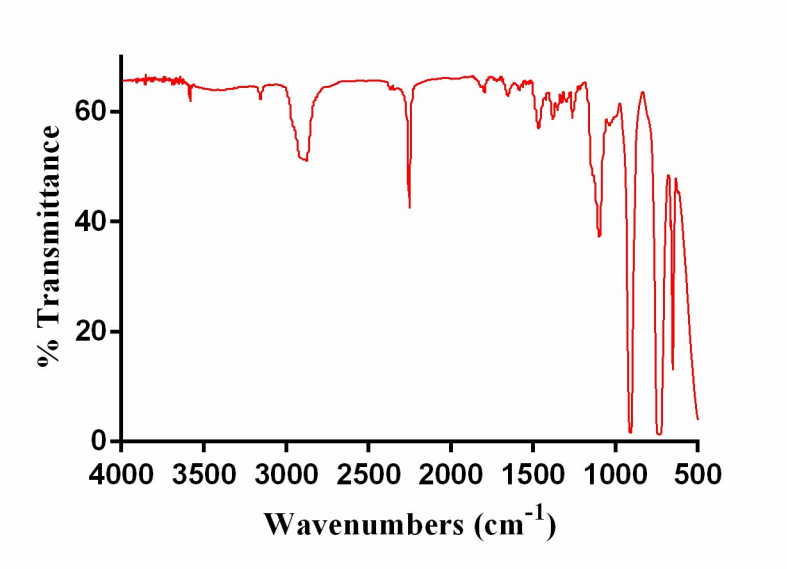

Figure S9. FT-IR spectrum of $mPEG_{2k}$ triallyl ether **(4)**.

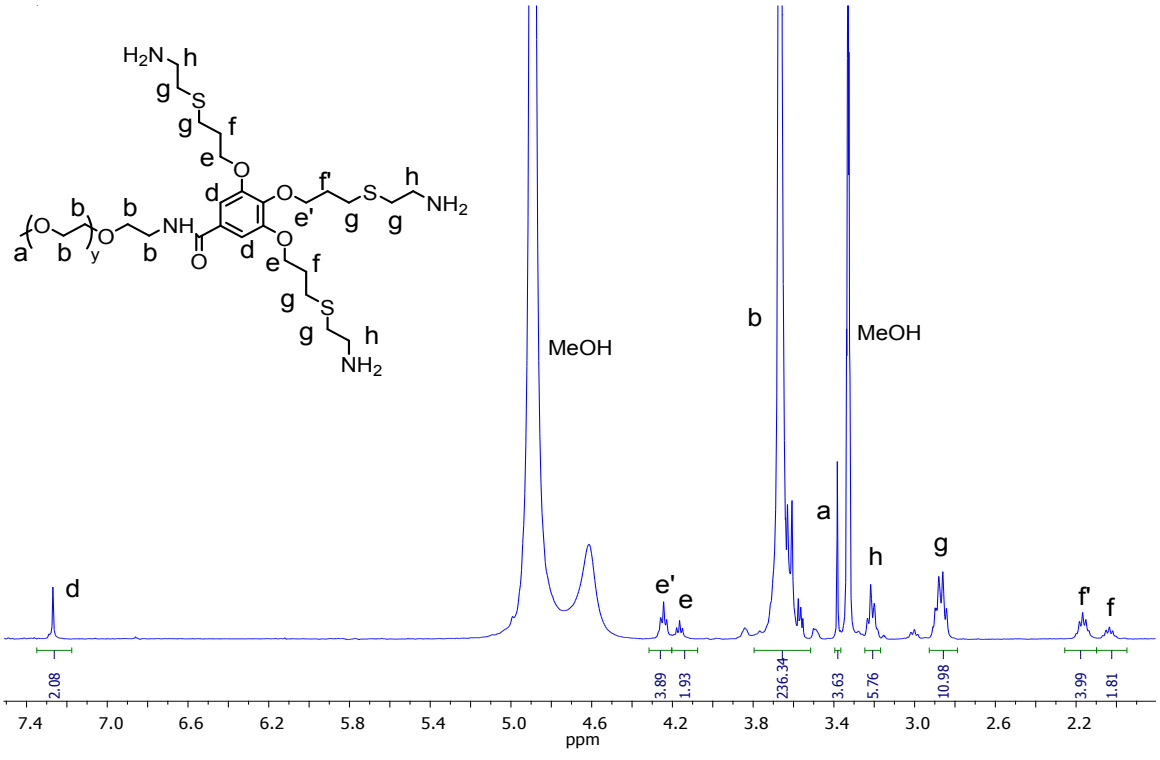

Figure S10. ^1H NMR spectrum of $triaminoPEG_{2k}$ **(5)** in CD_3OD.

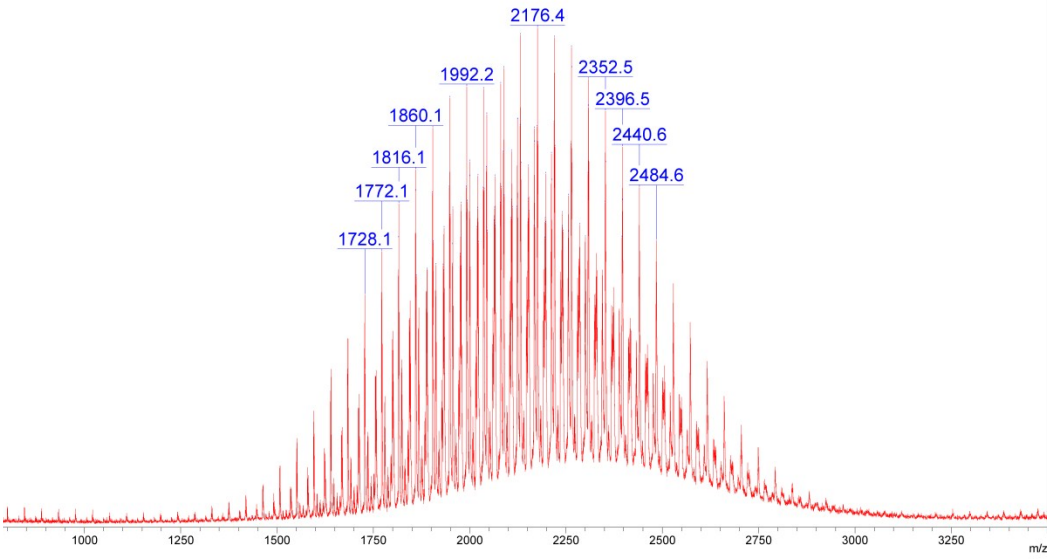

Figure S11. Mass spectrum (MALDI-TOF) of methoxypolyethylene glycol amine 2,000.

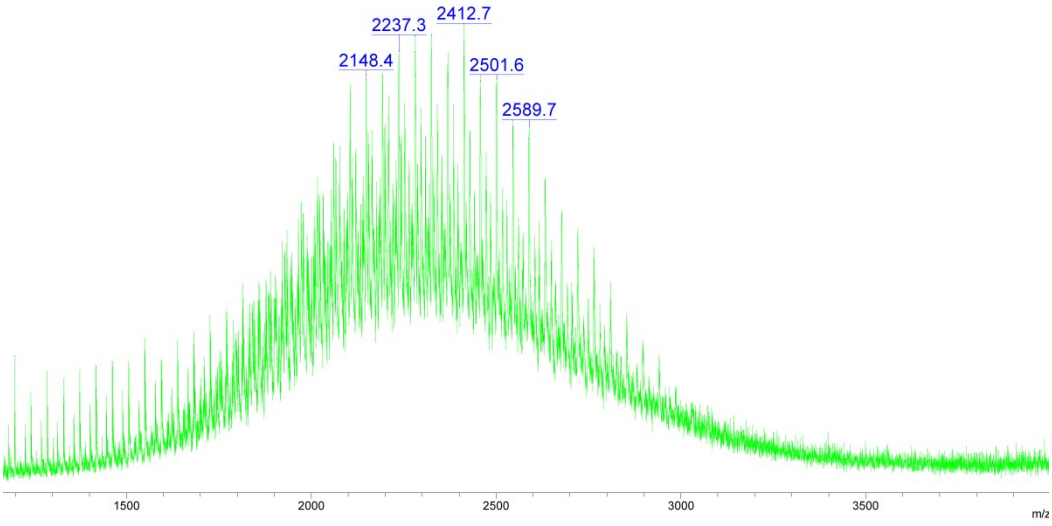

Figure S12. Mass spectrum (MALDI-TOF) of $mPEG_{2k}$ triallyl ether **(4)**.

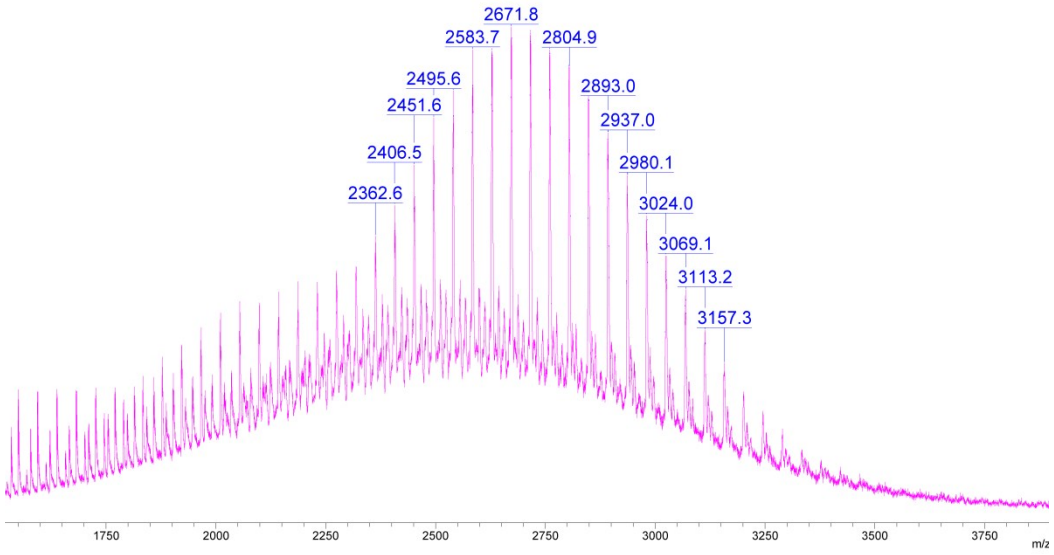

Figure S13. Mass spectrum (MALDI-TOF) of triaminoPEG$_{2k}$ **(5)**.

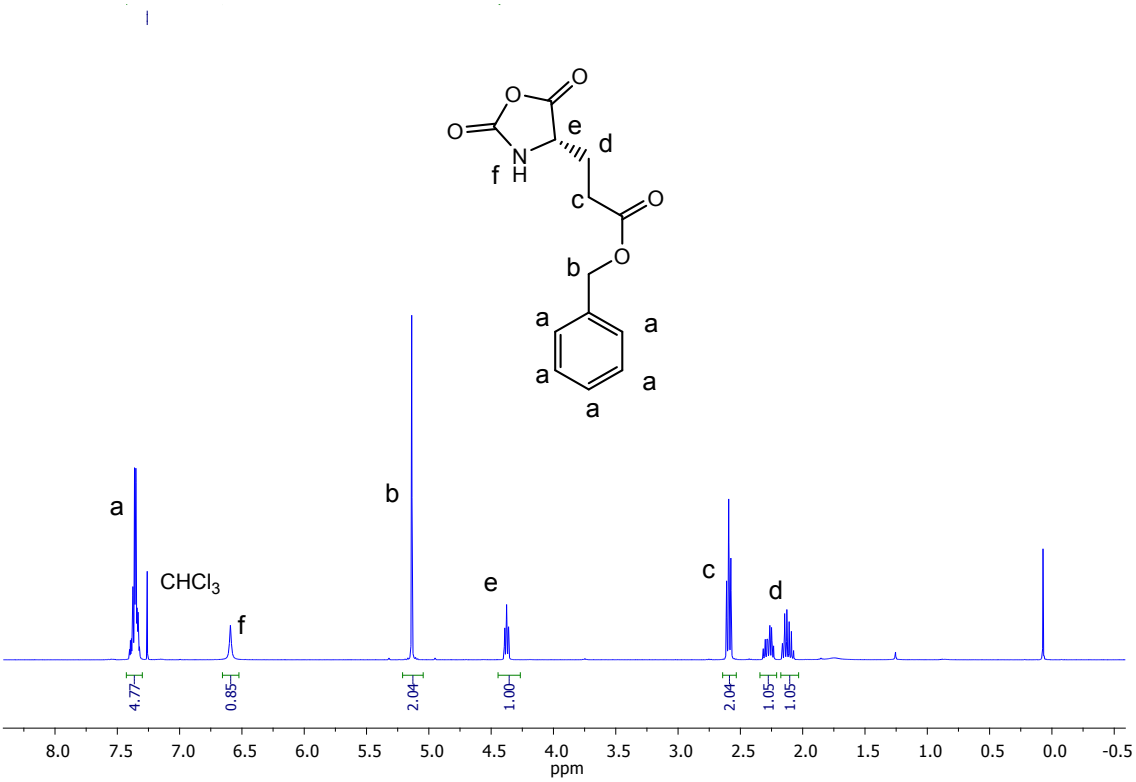

Figure S14. ^{1}H NMR spectrum of γ-benzyl-L-Glutamate *N*-carboxyanhydride (NCA) in $CDCl_3$.

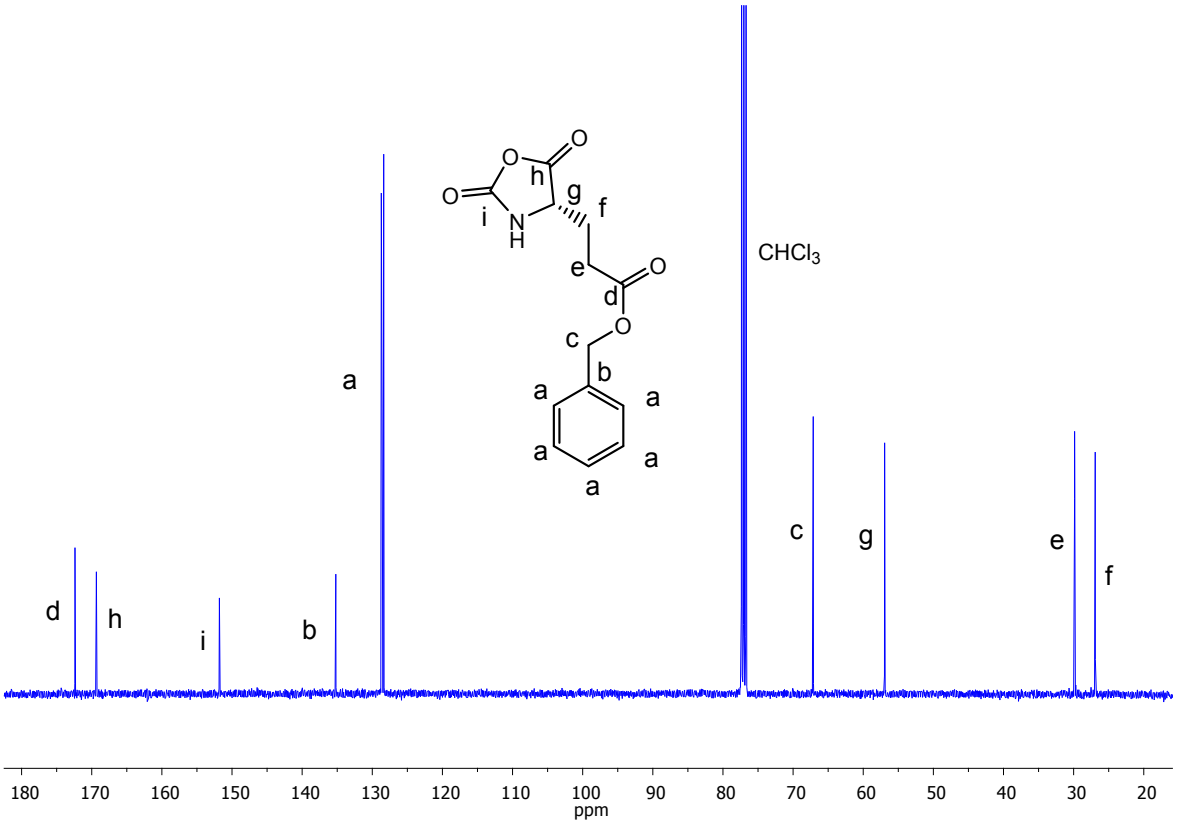

Figure S15. ^{13}C NMR spectrum of γ-benzyl-L-glutamate *N*-carboxy-anhydride (NCA) in $CDCl_3$.

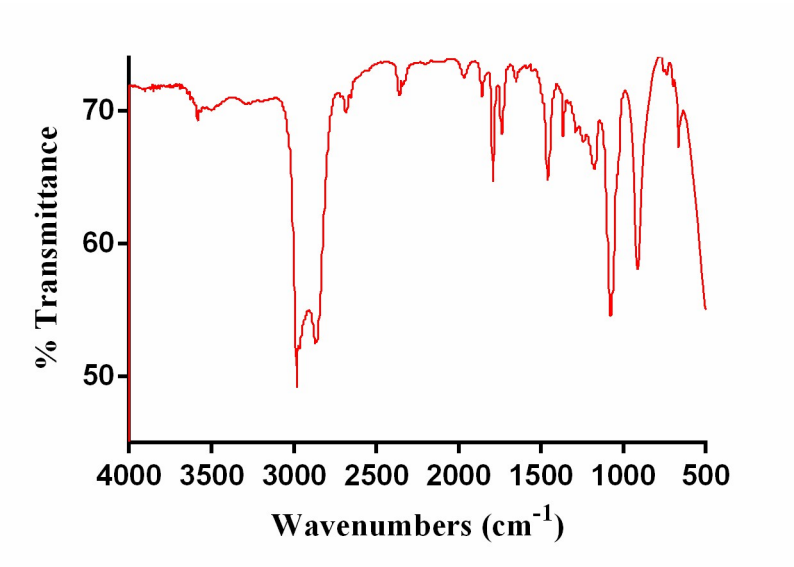

Figure S16. FT-IR spectrum of γ-benzyl-L-glutamate *N*-carboxy-anhydride (NCA).

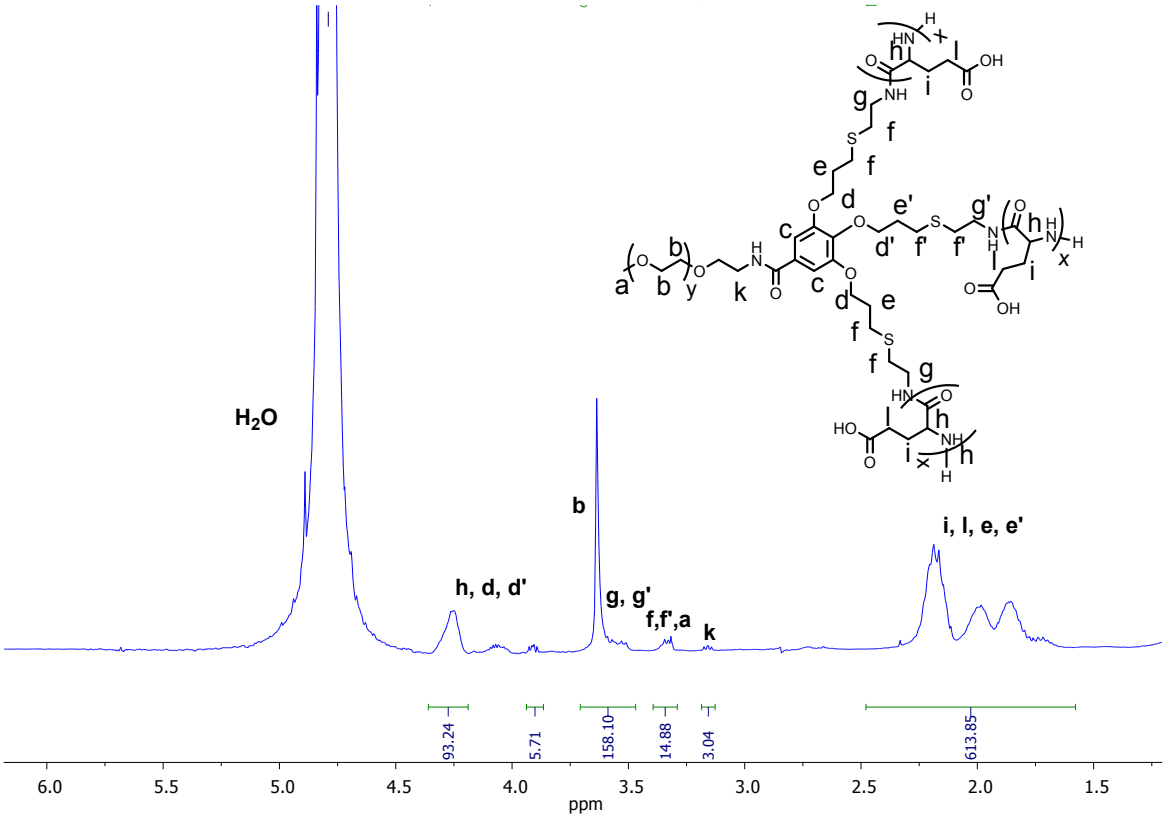

Figure S17. ^{1}H NMR spectrum of miktoarm mPEG$_{2k}$-*mik*-(GA$_{30}$)$_3$ copolymer **(6)** in D$_2$O.

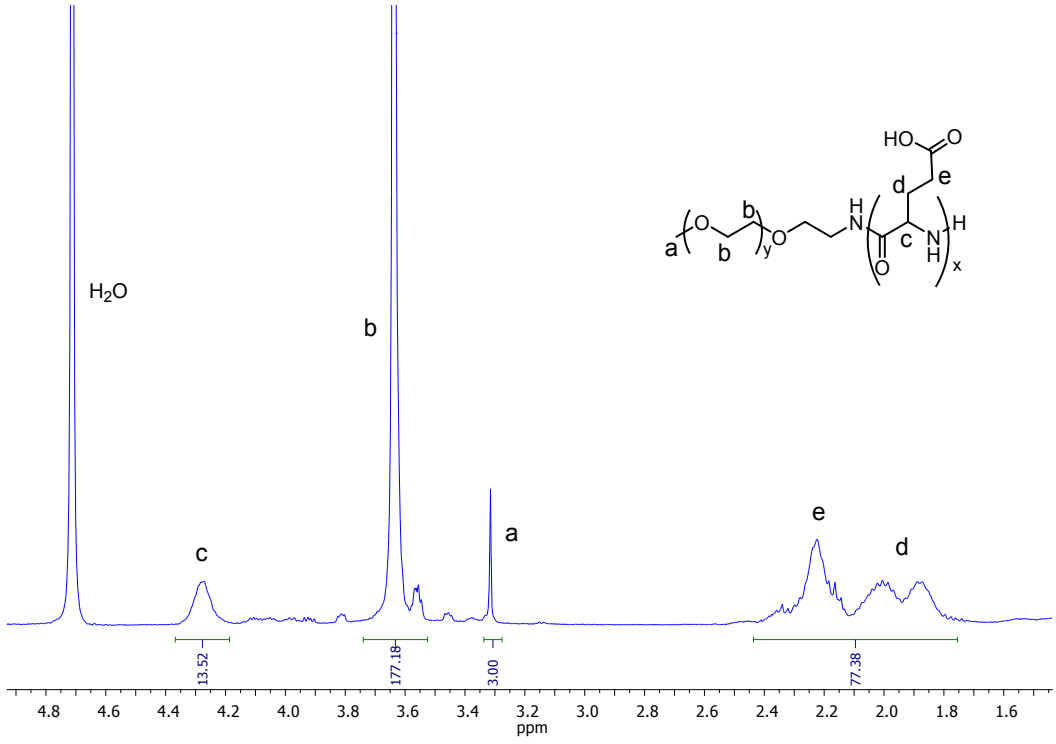

Figure S18. ^{1}H NMR spectrum of linear mPEG$_{2k}$-*lin*-GA$_{10}$ copolymer **(7)** in D$_2$O.

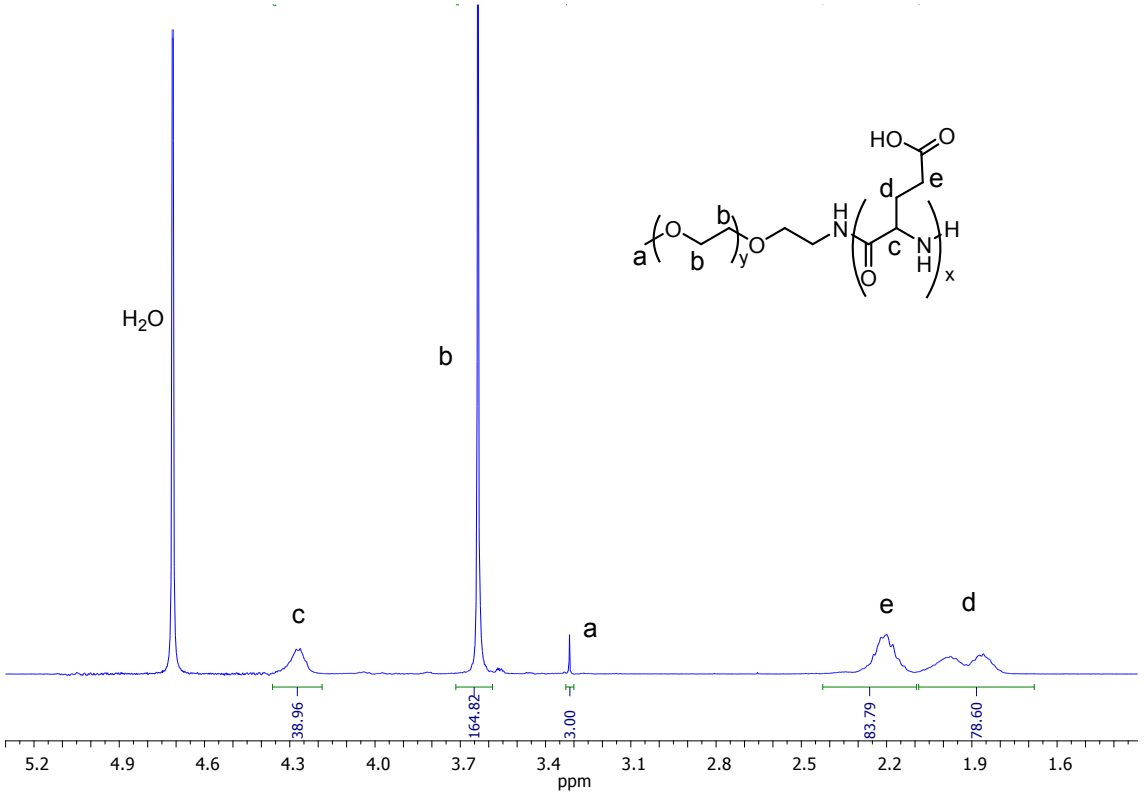

Figure S19. ^{1}H NMR spectrum of linear mPEG$_{2k}$-*lin*-GA$_{30}$ copolymer **(7)** in D_2O.

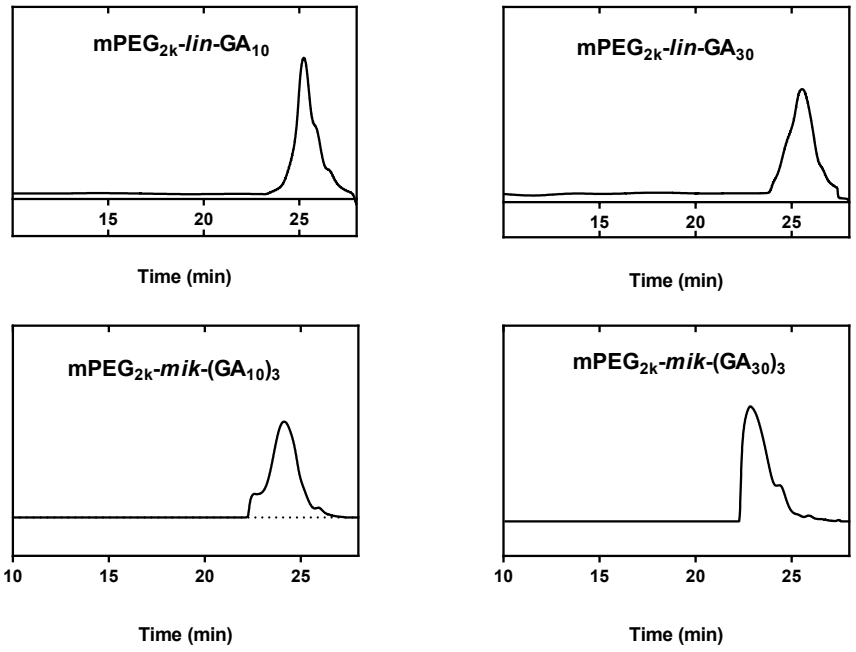

Figure S20. SEC chromatograms of linear mPEG$_{2k}$-*lin*-GA$_{10}$, mPEG$_{2k}$-*lin*-GA$_{30}$ and miktoarm mPEG$_{2k}$-*mik*-(GA$_{10}$)$_3$, mPEG$_{2k}$-*mik*-(GA$_{30}$)$_3$ copolymers in aqueous mobile phase 0.10 M $NaNO_3$, 0.02 (w/v) NaN_3, pH 10.

4. Circular dichroism

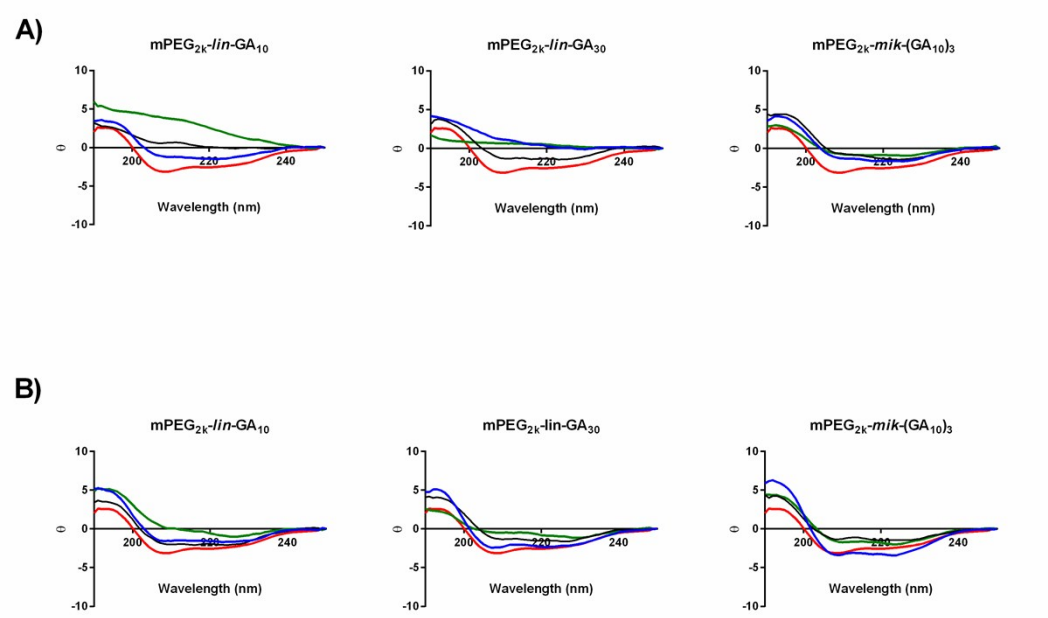

Figure S21. Circular dichroism spectra for free linear mPEG$_{2k}$-*lin*-GA$_{10}$, mPEG$_{2k}$-*lin*-GA$_{30}$ and miktoarm mPEG$_{2k}$-*mik*-(GA$_{10}$)$_3$ copolymers (A) and their respective complexes with lysozyme (B). Complexes were prepared at *r*: 2.5. Free lysozyme (red), *r*: 0.1 (blue), *r*: 1 (black) and *r*: 2.5 (green).

5. TEM analysis

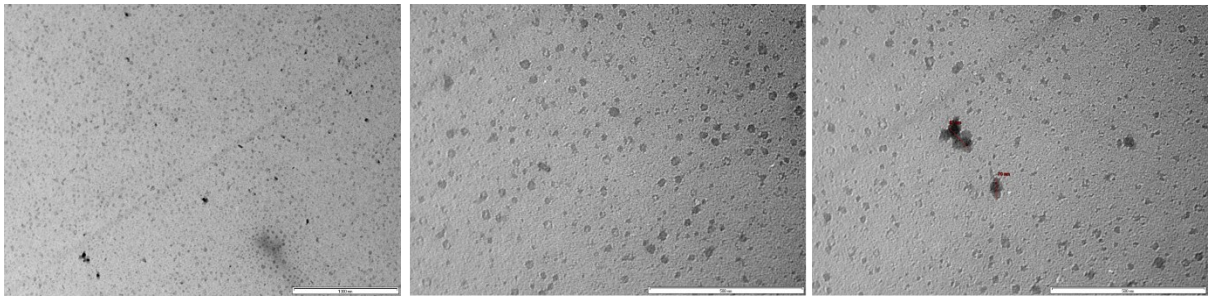

Figure S22. TEM images of lysozyme-mPEG$_{2k}$-*lin*-GA$_{30}$ complexes prepared at *r*: 2.5, as described in Experimental section. Scale bar 1000 nm, left and 500 nm, middle and right images.

6. Synthesis of mPEG-lysozyme covalent conjugates

Lysozyme (10 μmol) was dissolved in 10 mM phosphate buffer pH 8.0 (14.3 mL) and different amounts of methoxy PEG succinimidyl carbonate 1.9 kDa (50, 100, 200, or 300 μmol) were added to the solution, as per previous protocol [1]. Final protein concentration, calculated by BCA assay (Thermo Fisher), was always kept constant to 10 mg mL^{-1}. The reaction was kept under slow agitation for 1 day, and PEGylated proteins were purified by dialysis (MWCO: 10.0 kDa) against 10 mM phosphate buffer pH 7.4.

The number of conjugated PEG polymer chains *per* lysozyme was estimated by titrating the residual lysozyme primary amino groups (unmodified chicken egg white lysozyme possess 6 lysine residues and 1 *N*-terminal amine) by TNBS assay. Accordingly, the dialysed PEGylated protein solutions were incubated with 410 μL of 0.1 M borate buffer (pH 9.0) and 0.1 % (v/v) TNBS reagent in the dark, at room temperature for 30 minutes. The absorbance was recorded at λ=420 nm using a TECAN microplate reader, and the number of unreacted amino groups was calculated from a calibration curve built using unmodified lysozyme at different concentrations. A final 65, 74, 89 or 90 % of lysozyme amino groups – corresponding to 4.5, 5.2, 6.2, 6.3 mPEG chains *per* protein - were found to be reacted following incubation with 5, 10, 20 or 30 $mPEG_{2k}$ equivalents, respectively (Table S1).

Table S1. Percentage of lysozyme amino groups reacted with $mPEG_{2k}$, as estimated by TNBS assay.

$mPEG_{2k}$:Lysozyme molar ratio in the reaction feed	Percentage of lysine reacted with PEG (%)	Average number of $mPEG_{2k}$ per lysozyme
5	65	4.5
10	74	5.2
20	89	6.2
30	90	6.3

SDS-PAGE was performed using a BioRad mini-PROTEAN Tetra Cell. The sample was prepared under reducing conditions for application on a gel consisting of 6 and 15 % stacking and resolving gel, respectively. Coomassie blue staining was used to visualize the protein bands.

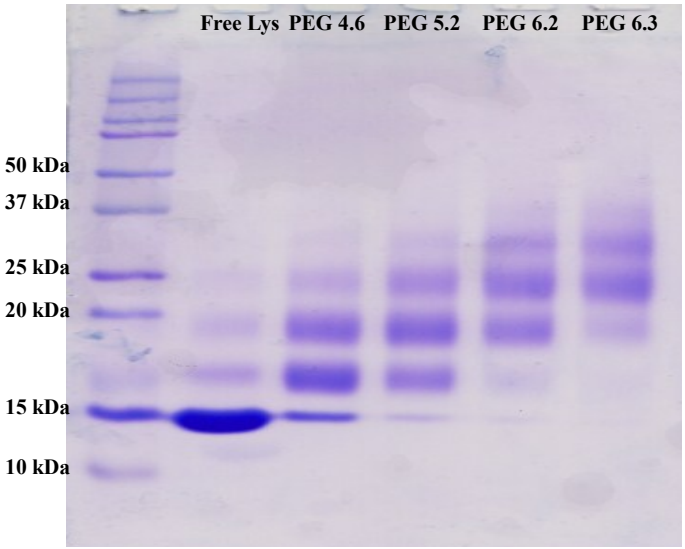

Figure S23. SDS-PAGE of PEGylated lysozyme at different $mPEG_{2k}$: lysozyme molecular average ratios.

The enzymatic activity of the synthesized PEGylated lysozyme conjugates was evaluated. Lysozyme-$(mPEG_{2k})_{4.6}$ conjugate sample was not utilised in this part of our study because it still contained a non-negligible amount of unmodified enzyme. A lysozyme concentration of 37 µg mL^{-1} was utilised in all enzymatic assay tests. 10 mM phosphate buffer pH 7.4 (20 mL) was added to 10 mg of *Micrococcus lisodeikticus* lyophilised cells. 300 µL of the resulting suspension was added to 150 µL of nanocomplexes suspension and the decrease in optical density at λ = 460 nm measured as a function of time. All enzyme digestion experiments were carried out in triplicate. Data are presented in Figure S24.

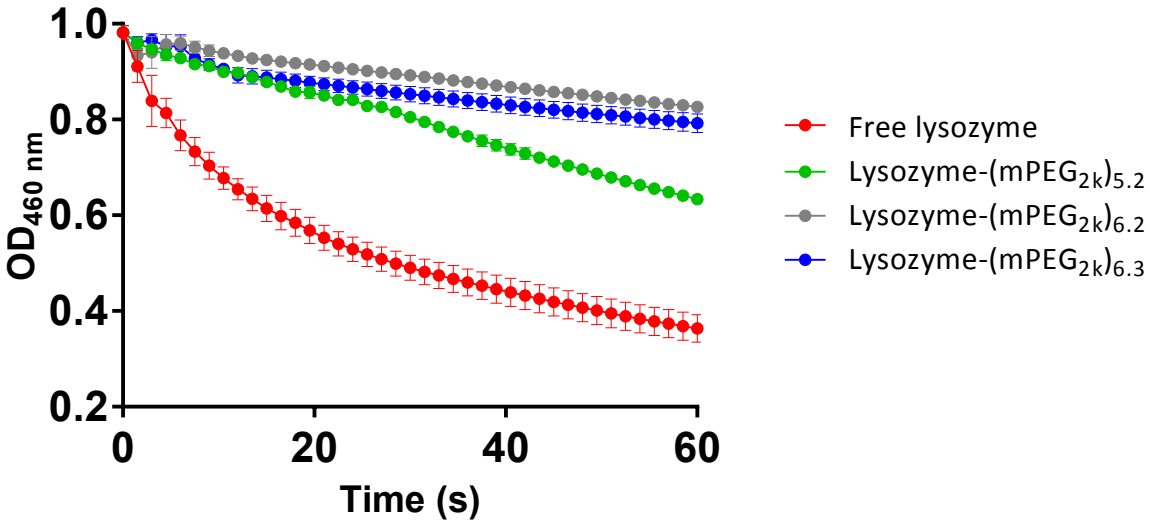

Figure S24. Enzymatic activity of lysozyme-$(mPEG_{2k})_{5.2}$, lysozyme-$(mPEG_{2k})_{6.2}$ and lysozyme-$(mPEG_{2k})_{6.3}$ PEGylated proteins containing different amounts of $mPEG_{2k}$ chains *per* lysozyme molecule. All experiments were carried out in triplicate.

6. References.

1. T. Miron and M. Wilchek, *Bioconjugate Chemistry*, 1993, **4**, 568-569.