Supporting Information

A novel acid-degradable PEG crosslinker for the fabrication of pH-responsive soft materials

Dr. Iuliia Myrgorodska, Mary Jenkinson-Finch, Rafael Moreno Tortolero, Prof. Stephen Mann and Dr. Pierangelo Gobbo**

Table of Contents

1. Supplementary Methods 2

1.1. Materials and general procedures 2

1.2. Synthesis of acid-degradable crosslinker 4

1.2.1. Synthesis of 2,2-Bis(phthalimidoethoxy)propane (**1**) 5

1.2.2. Synthesis of 2,2-Bis(aminoethoxy)propane (**2**) 5

1.2.3. Synthesis of Bis(succinic acid ester)polyethylene glycol (**3**) 5

1.2.4. Synthesis of O,O'-Bis[2-(N-Succinimidyl-succinate]polyethylene glycol (**4**) 5

1.2.5. Synthesis of 2,2-Bis({O-[(N-Succinimidyl)succinyl]-O'-[succinylamino]polyethylene glycolethoxy)propane (**5**) 6

*1.3. Hydrolysis kinetics of acid-degradable crosslinker (**5**) 6*

1.4. Preparation of acid-degradable chemically crosslinked CM-chitosan hydrogels 7

1.5. Acid-mediated degradation of CM-chitosan hydrogels 7

1.6. Synthesis of RITC-labelled BSA/PNIPAm nanoconjugates 8

1.7. Preparation of acid-degradable hydrogel-filled proteinosomes 9

1.8. Time-dependent fluorescence microscopy studies of acid-mediated proteinosome degradation 10

1.9. Time-dependent FACS analysis of acid-mediated proteinosome degradation 10

2. Supplementary Figures 11

3. Supplementary Videos 25

4. Supplementary References 25

1. Supplementary Methods

1.1. Materials and general procedures

The following reagents and solvents were purchased and used as received. Albumin from bovine serum (BSA, isoelectric point = 4.6) (≥98%, Mw ~66 kDa), 1,6-diaminohexane (hexamethylenediamine, HMDA 98 %), *N*-ethyl-*N*'-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDAC) (98%), *N*,*N*'-dicyclohexylcarbodiimide (DCC) (99%), fluorescein 5(6)-isothiocyanate (FITC, >90%), rhodamine B isothiocyanate (RITC), Glucose Oxidase from *Aspergillus niger* (GOx, 224890 U/g), D-Glucose (>99%), L-Glucose (>99%), 4-dimethylaminopyridine (DMAP) (>99%), triethylamine (TEA) (>99%), 2,2-dimethoxypropane, *p*-toluenesulfonic acid monohydrate (TsOH), 5 Å molecular sieves, DMF (anhydrous, 99.8%), deuterated methanol (CD_3OD) (99.8 atom %) and deuterated chloroform ($CDCl_3$) (99.8 atom %), *O*,*O*'-Bis[2-(*N*-Succinimidyl-succinylamino)ethyl]polyethylene glycol (M_n = 2000 Da, PEG-diNHS), 2-ethyl-1-hexanol, α-cyano-4-hydroxycinnamic acid (CHCA), 2,6-dihydroxyacetophenone (DHAP), diammonium hydrogen citrate (DAHC), trifluoroacetic acid (TFA), were purchased from Sigma-Aldrich. Succinic anhydride and poly(ethylene glycol) (M_n = 1000 Da) were purchased from Merck. Universal Indicator Solution pH 3 – 10 was purchased from Honeywell Fluka™. *N*-hydroxysuccinimide (NHS) (98+%) and *N*-(2-hydroxyethyl)phthalimide were purchased from Alfa Aesar. Sodium hydroxide (NaOH), sodium carbonate (>98%) were acquired from Fischer Scientific. Carboxymethyl chitosan (CM-chitosan, deacetylation degree 90%) was purchased from Santa Cruz Biotechnology. Dialysis bags with MWCO 1,000 or 12,000–14,000 Da were purchased from Millipore.

^1H and ^{13}C NMR spectra were obtained using Varian 400 MHz or 500 MHz spectrometers, indicated below each NMR spectra shown. ^1H NMR spectra are reported as δ in units of parts per million (ppm) relative to chloroform (δ 7.26, s). Multiplicities are reported as follows: s (singlet), d (doublet), t (triplet), q (quartet), p (quintuplet), and m (multiplet). Coupling constants are reported as *J* values in units of Hertz (Hz). The number of protons (*n*) is reported as *n*H, and based on spectral integration values. ^{13}C NMR are reported as δ in units of parts per million (ppm) relative to $CDCl_3$ (δ 77.16, t).

MALDI spectra were recorded on Bruker UltrafleXtreme (TOF-TOF). For polyethylene glycol samples: α-cyano-4-hydroxycinnamic acid (7 mg mL^{-1} in CH_3CN/0.1 vol% TFA) was used as the matrix solution. The matrix solution was mixed with the polymer sample (1-2 mg mL^{-1} in CH_3CN/0.1 vol% TFA) in a 1:1 v/v ratio. For proteins and protein-polymer nanoconjugate samples, the matrix solution was made using 3 equivalents 2,6-dihydroxyacetophenone in EtOH (20.3 mg mL^{-1}) and 1 equivalent diammonium hydrogen citrate in water (18 mg mL^{-1}). To prepare the sample, 1:1:1 v/v mixture of protein in water (1 mg mL^{-1}), 2% trifluoracetic acid solution in water, and a matrix solution was used.

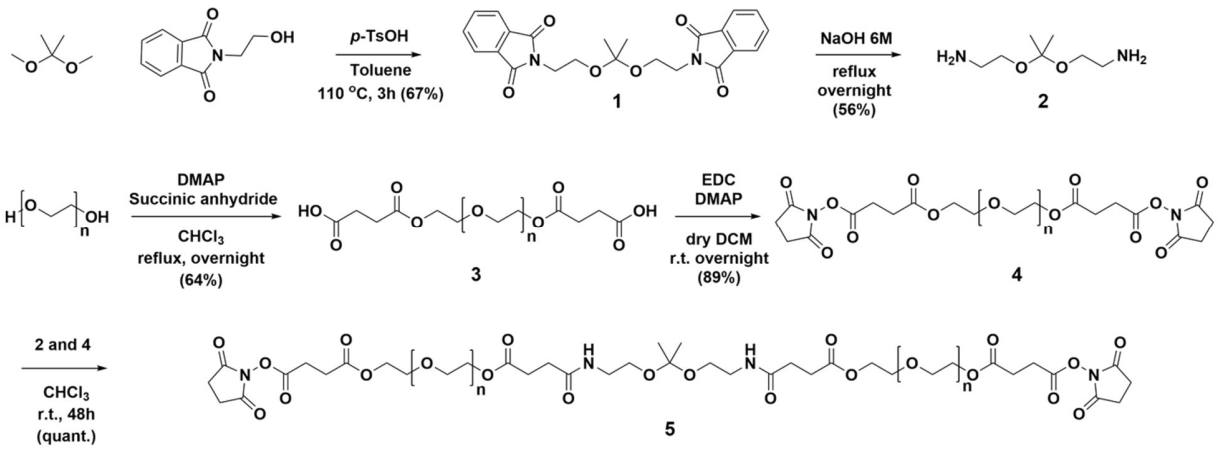
Optical and fluorescence microscopy was performed on a Leica DMI3000 B manual inverted fluorescence microscope at ×20 and ×40 magnification. Time-lapse images were taken at intervals of 1 min. Images were analyzed using ImageJ software.

Confocal microscopy images were obtained on a Leica SP8 AOBS confocal laser scanning microscope attached to a Leica DM I6000 inverted epifluorescence microscope. The microscope was equipped with the following lasers: 65 mW Ar (458, 476, 488, 496 and 514 nm lines), 20 mW solid state yellow (561 nm), 2 mW Orange HeNe (594 nm), 10 mW Red He/Ne (633 nm) and a 50 mW 405 nm diode. All measurements were performed in an environmental chamber maintained at 25 °C. Images were analyzed using ImageJ software.

Zeta potential studies of the protein-polymer nanoconjugate solutions in pre-filtered MilliQ water (1.0 mg mL^{-1}) were carried out at 25°C using a ZETASIZER Nano series instrument (Malvern Instruments, UK).

Rheology experiments were performed using a Malvern Kinexus Pro Rheometer (Malvern Instruments Ltd, Malvern, UK) equipped with a parallel plate geometry (diameter of 20 mm), and a Peltier heated lower plate for temperature control. For the initial strain and frequency sweeps hydrogel samples were prepared by mixing CM-chitosan (40 mg mL^{-1}, 1.5 mL) with 15 mg of crosslinker (**5**) directly inside a circular mold 22 mm in diameter and with a mobile bottom. Samples were let to crosslink for 2 h and then placed on the base plate of the rheometer by slowly removing the mold's bottom. The gap was set using the normal force control, and plate was stopped at the value of 0.5 N. All measurements were performed at 25 °C using a freshly prepared hydrogel sample. An initial strain sweep from 0.01 to 2000% strain was then conducted at a frequency of ω_0 = 1.0 Hz (6.3 rad s^{-1}) on the fully formed gel to determine the linear elastic limit. Frequency sweeps from 0.01 to 10 Hz were later conducted on fresh samples prepared as described above at a set strain of 10% to prove the independency of the properties from frequency; frequency was limited to 10 Hz. As frequency value reached over 10 Hz, the moduli became dependent on the frequency, *i.e.,* viscoelastic effects dominated over the microstructural parameters of the gel. Finally, gelation kinetics was determined for hydrogel samples prepared by mixing CM-chitosan (40 mg mL^{-1}, 1.5 mL) with different amounts of crosslinker (5) ranging between 15-40 mg directly inside a mold (see above). Time sweeps were conducted at 10% strain and 1 Hz frequency (both values within the linear viscoelastic behavior of all samples) over 3,000 s. Gelation time point was determined as the time when *G'* and *G''* are equal; alternatively, when tanδ = 1.

1.2. Synthesis of acid-degradable crosslinker



Scheme S1. Synthetic route to the preparation of the acid-degradable crosslinker **5**.

The synthesis of the PEG-based acid-degradable crosslinker consisted of five steps (**Scheme S1**). We started with the synthesis of an acid-degradable ketal-diamine core (**2**) by reacting commercially available 2,2-dimethoxypropane with *N*-(2-hydroxyethyl)phthalimide in dry toluene; residual water was removed by azeotropic distillation in a Dean-Stark distillation setup (**Figure S1**). Phthalimide protected intermediate (**1**) was subsequently deprotected upon reflux with sodium hydroxide to obtain 2,2-bis(aminoethoxy)propane (**2**) in high purity. Significantly, the ^{1}H NMR spectrum of compound (**2**) showed the typical singlet of the ketal group at 1.37 ppm (**Figure S2**). Separately, the terminal hydroxy groups of polyethylene glycol (M_n 1,000 Da) were transformed into carboxylic acids *via* the addition of succinic anhydride in the presence of a strong organic base. The reaction was carried out under anhydrous conditions and under an inert atmosphere, and the dicarboxylic acid product (**3**) was purified by dialysis with 1,000 Da MWCO membrane. The formation of the desired bis(succinic acid ester)polyethylene glycol (**3**) was confirmed by ^{1}H NMR spectroscopy by the appearance of a broad peak at 4.24 ppm due to the methylene alpha to the ester group, and by the appearance of a new signal at 2.63 ppm, which corresponded to the methylene groups of the 2 succinic acid moieties (**Figure S3**). The MALDI-TOF spectra of polymer (**3**) indicated that the M_n of the polymer was 1250 Da, in good agreement with the addition of the 2 succinic acid groups to the two ends of the polymer chain (**Figure S4**). The terminal carboxylic groups of polymer (**3**) were then activated with NHS *via* an EDC-mediated coupling reaction in the presence of a strong organic base to obtain *O*,*O*'-bis[2-(*N*-succinimidyl-succinate]polyethylene glycol (**4**). The successful synthesis of PEG-diNHS (**4**) was confirmed by the appearance of a singlet at 2.84 ppm and of 2 triplets at 2.78 and 2.95 ppm in the ^{1}H NMR spectrum of the product, associated with the methylene protons of the terminal NHS groups and the methylene groups of the NHS-activated succinic acid, respectively (**Figure S5**). It was also confirmed by a further increase in the polymer's M_n from 1250 Da to 1450 Da (**Figure S6**). PEG-diNHS (**4**) was then reacted with 2,2-bis(aminoethoxy)propane (**2**) in a 1:2 molar ratio to yield acid-degradable crosslinker (**5**) in quantitative yield. The successful reaction was confirmed by NMR spectroscopy and MALDI-TOF spectrometry. The ^{1}H NMR spectrum of compound (**5**) showed the appearance of the ketal peak at 1.33 ppm, of the methylene protons peak at 3.40 and 3.46 ppm and by a

2-fold increase of the PEG peak at 3.63-3.71 ppm (**Figures S7, S8**). MALDI-TOF mass spectrometry also confirmed coupling of 2 polymers (**4**) to 1 acid-degradable ketal-diamine core (**2**) (**Figure S9**). Crosslinker (**5**) was found to be stable for months if stored at –20 °C and under inert atmosphere and was used directly in the subsequent experiments without any additional purification steps.

1.2.1. Synthesis of 2,2-Bis(phthalimidoethoxy)propane (**1**)

Compound (**1**) was synthesized following the previously reported procedure.[1] *N*-(2-hydroxyethyl)phthalimide) (5 g, 26 mmol, 2 eq.) was dissolved in 200 mL of dry toluene; residual water was removed by azeotropic distillation in a Dean-Stark distillation setup. The solution was cooled to room temperature and 2,2-dimethoxypropane (1.58 mL, 13 mmol, 1 eq.) was added to the solution along with *p*-toluenesulfonic acid (55 mg). The reaction mixture was heated to 110 °C for 3 h. Finally, the reaction mixture was cooled to room temperature and triethylamine (TEA) (6 mL) was added to quench the reaction. To facilitate further purification, acetic anhydride (2 mL) was added to convert any unreacted alcohol groups into the corresponding acetate and the reaction mixture was allowed to stir overnight. The product (**1**) was then precipitated by dropwise addition into a large excess of hexanes. The precipitated powder was collected and recrystallized from ethyl acetate and the excess solvent was removed *in vacuo* to yield (**1**) (3.7 g, 8.76 mmol, 67%). ^{1}H NMR (400 MHz, $CDCl_3$) δ_H 7.82 (m, 4H, Ar-H), 7.67 (m, 4H, Ar-H), 3.81 (t, 4H), 3.59 (t, 4H), 1.25 (s, 6H). ^{13}C NMR (400 MHz, $CDCl_3$) δ_C 168.2,134.1, 133.9, 132.1, 123.3, 100.6, 57.9, 38.1, 24.7. HRMS (ESI): calc. for $C_{23}H_{22}N_2NaO_6^+$ [M+Na] 445.1370, found 445.1375.

1.2.2. Synthesis of 2,2-Bis(aminoethoxy)propane (**2**)

2,2-Bis(phthalimidoethoxy)propane (**1**) (2.0 g, 4.7 mmol) was re-dispersed in 6 M NaOH (60 mL) and refluxed overnight. The mixture was then diluted with water and extracted 3 times with dichloromethane. The combined organic layers were dried over anhydrous Na_2SO_4 and evaporated to yield (**2**) as a thick yellow oil (263 mg, 1.62 mmol, 56% yield). ^{1}H NMR (400 MHz, $CDCl_3$) δ_H 3.46 (t, 4H), 2.84 (t, 4H), 1.37 (s, 6H). ^{13}C NMR (400 MHz, $CDCl_3$) δ_C 99.8, 62.9, 42.0, 25.0. HRMS (ESI): calc. for $C_7H_{19}N_2O_2^+$ [M+H] 163.1441, found 163.1446.

1.2.3. Synthesis of Bis(succinic acid ester)polyethylene glycol (**3**)

To a round bottom flask pre-loaded with DMAP (0.2 g, 1.6 mmol) was added poly(ethylene glycol) $(PEG)_{23}$ (MW 1000 Da, 4.0 g, 4.0 mmol) under inert atmosphere. The solids were dissolved in chloroform (100 mL), followed by the addition of succinic anhydride (1.2 g, 12.0 mmol). The reaction mixture was refluxed overnight. The solvent was removed *in vacuo* and the residue was redissolved in water. The final product was dialyzed against MillQ water using a 1 kDa MWCO membrane overnight and lyophilized to yield (**3**) (3.2 g, 2.56 mmol, 64 %). ^{1}H NMR (400 MHz, $CDCl_3$) δ_H 4.24 (t, 4H), 3.68-3.63 (m, 95H), 2.63 (tt, 8H). ^{13}C NMR (400 MHz, $CDCl_3$) δ_C 174.8, 172.2, 70.6, 70.5, 69.0, 63.9, 29.3, 29.0. M_n (MALDI-TOF MS) = 1250 Da.

1.2.4. Synthesis of O,O'-Bis[2-(N-Succinimidyl-succinate]polyethylene glycol (**4**)

Bis(succinic acid ester)polyethylene glycol (**3**) (M_n = 1250, 2.0 g, 1.6 mmol), DMAP (50 mg, 0.41 mmol) and NHS (552 mg, 4.8 mmol) were dissolved in dry dichloromethane (25 mL) and the solution was cooled to

0 °C. This was followed by the dropwise addition of EDC (744 mg, 4.8 mmol) dissolved in 5 mL dry dichloromethane. The reaction mixture was stirred overnight at room temperature. The urea by-product was removed through aqueous extraction. The organic layer was dried with sodium sulfate and concentrated *in vacuo*. The polymer was precipitated from cold diethyl ether. The organic layer was separated by centrifugation and decantation, and the solvent excess was removed *in vacuo* yielding (**4**) (2.06 g, 1.42 mmol, 89%). ^{1}H NMR (400 MHz, $CDCl_3$) δ_H 4.27 (t, 4H), 3.71-3.63 (m, 92H), 2.95 (t, 4H), 2.84 (s, 8H), 2.78 (t, 4H). ^{13}C NMR (400 MHz, $CDCl_3$) δ_C 171.0, 169.0, 167.8, 70.7, 70.6, 69.0, 64.3, 28.7, 26.3. 25.6. M_n (MALDI-TOF MS) = 1450 Da.

1.2.5. Synthesis of 2,2-Bis({O-[(N-Succinimidyl)succinyl]-O'-[succinylamino]polyethylene glycolethoxy)propane (**5**)

O,*O*'-Bis[2-(*N*-Succinimidyl-succinate]polyethylene glycol, Mn=1450 (**4**) (3.48 g, 2.4 mmol) was dissolved in chloroform (100 mL). Separately, di-amino ketal (**2**) (200 mg, 1.2 mmol) was dissolved in chloroform (100 mL) and was added dropwise to the stirring PEG solution. The reaction mixture was stirred at room temperature and under a normal atmosphere for 48 hours. The solvent was removed *in vacuo* to yield the final crosslinker (**5**). The product (**5**) was used in following experiments without further purification. ^{1}H NMR (500 MHz, $CDCl_3$) δ_H 4.27 (t, 4H), 4.22 (t, 4H), 3.71-3.63 (m, 182H), 3.46 (t, 4H), 3.40 (t, 5.57), 2.95 (t, 4H), 2.84 (s, 8H), 2.78 (t, 4H), 2.71 (s, 8H), 2.50 (m, 4H), 1.33 (s, 6H). ^{13}C NMR (500 MHz, $CDCl_3$) δ_C 172.1, 171.0, 169.0, 167.7, 100.2, 70.6, 70.5, 68.9, 64.2, 63.8, 59.5, 39.7, 30.8, 29.5, 28.7, 26.3, 25.6, 24.8. M_n (MALDI-TOF MS) = 2800 Da.

1.3. Hydrolysis kinetics of acid-degradable crosslinker (**5**)

Hydrolysis kinetics experiments were performed at different pH ranging from 5 to 8.5. In general, acid-degradable crosslinker **5** (20 mg) was dissolved in a deuterated phosphate buffer (PBS, 100 mM, 500 μL) of appropriate pH. The pH was then measured and, if needed, adjusted to the appropriate pH value using an aqueous NaOH solution (5 M). Hydrolysis kinetics of acid-degradable crosslinker (**5**) was followed by ^{1}H NMR spectroscopy by acquiring spectra at different time intervals on a Varian 500 MHz spectrometer using a pre-acquisition delay parameter of 1 min or 5 min for samples in acidic or basic pH, respectively. The acquired spectra were analyzed by integrating the ketal peak (δ 1.33, s, 6H) and the acetone peak (δ 2.22, s, 6H). The percentage hydrolysis at a given time was calculated according to the following equation:

$$\%\, hydrolysis = \frac{I_{acetone}}{(I_{ketal}+I_{acetone})} \times 100\% \qquad (1)$$

Where $I_{Aacetone}$ is the integral of the acetone peak, and I_{Ketal} is the integral of the ketal peak.

The hydrolysis kinetics of acid-degradable crosslinker (**5**) follows a pseudo first-order kinetics.

$$[Ketal]_t = [Ketal]_0 e^{-k_{obs}t} \qquad (2)$$

Where $[Ketal]_t$, is the concentration of ketal crosslinker (**5**) at time *t*, which is proportional to I_{Ketal} derived from the time-dependent ^{1}H NMR spectroscopy measurement, and $[Ketal]_0$ is the initial concentration of acid-degradable crosslinker (**5**) (0.014 M). Equation *(2)* can be re-written by taking the natural log of both sides to yield a linear plot where the slope is equal to k_{obs}.

$$\ln\left(\frac{[Ketal]_t}{[Ketal]_o}\right) = -k_{obs}t \quad (3)$$

Or, using the integrals obtained from the ^{1}H NMR spectra:

$$\ln\left(\frac{I_{ketal}}{I_{acetone}+I_{ketal}}\right) = -k_{obs}t \quad (4)$$

Figure S10 shows the k_{obs} values determined as a negative value of the slope obtained through the linear fitting of the plot $\ln\left(\frac{I_{ketal}}{I_{acetone}+I_{ketal}}\right)$ against incubation time *t* for the different kinetics carried out at different pH values. The half-life of hydrolysis of the acid-degradable crosslinker (**5**) was determined as follows:

$$t_{1/2} = \frac{\ln(2)}{k_{obs}} \quad (5)$$

1.4. Preparation of acid-degradable chemically crosslinked CM-chitosan hydrogels

Different hydrogel samples were prepared with a final concentration of crosslinker (**5**) ranging from 5, 12.5, 25, 50, and 100 mg mL^{-1} or 1, 2.5, 5, 10, and 20 mg by the total mass. In general, in a 1.75 mL vial, 190 μL of an aqueous solution of CM-chitosan (40 mg mL^{-1}, pH = 9.56), 5 μL of acid-degradable crosslinker (**5**) in Na_2CO_3 buffer solution (pH = 8.5, 0.1 M), and 5 μL of a commercial solution of universal indicator were mixed vigorously for 20 sec. Gelation was confirmed by turning the vial upside-down.

1.5. Acid-mediated degradation of CM-chitosan hydrogels

Degradation of the hydrogels prepared in **Supplementary section 1.4** was triggered by the direct addition of hydrochloric acid (5 μL, 1 M) to the vials containing the soft material. The vial was vortexed for 10s following the addition of HCl. The drop in pH was confirmed by the color change of the universal indicator, and gel degradation was monitored over 48 h. Corresponding photographs were taken with 1 h intervals for the first 10 h, and then at 24 h and 48 h (data shown in **Figure 2**).

Control experiments were performed in the same way with the exception that pH stable PEG-diNHS (M_n = 2000 Da) was used instead of acid-degradable crosslinker (**5**) (data shown in **Figure S14**).

A quantitative study of time-dependent hydrogel degradation was then carried out using the weighing method. For these experiments, 200 μL of CM-chitosan solution (40 mg mL^{-1}) was mixed with 3mg of crosslinker (**5**) in a pre-weighed Eppendorf tube. The hydrogel was let to set for 30 min and 300 μL of HCl 1 M was added into the Eppendorf tube. After a defined time interval (0.5, 1, 1.5, 2, and 2.5 hrs), excess liquid was removed from the Eppendorf tube with a pipette and the remaining liquid was absorbed using a soft paper tissue. The hydrogel was then freeze-dried and weighted. The experiment was run in triplicate. Gel degradation percentage was determined from the following equation:

$$GD = \frac{m_{tx}}{m_{t0}} \times 100 \quad (6)$$

Where GD is gel degradation, m_{t_x} is the mass of the freeze-dried hydrogel at time point t_x, and m_{t_0} is the mass of the freeze-dried hydrogel at t_0.

For the experiments of GOx-mediated gel degradation, in pre-weighed Eppendorf tubes, 200 μL of a CM-chitosan solution (40 mg mL^{-1}) was mixed with 3 mg of crosslinker (**5**) and 10 μL of a solution of GOx in MilliQ water (50 mg mL^{-1}). The hydrogel was let set for 30 min. Subsequently, 300 μL of D-Glucose (0.5 M in MilliQ water) were added into the Eppendorf tube. Reactions were stopped by removing all liquid from the Eppendorf tubes at different time intervals (0, 2, 4, 8, 12, 24, and 32 hrs). The remaining gel was freeze-dried and weighed. The experiment was run in triplicate.

A set of control experiments was run in parallel under the same conditions and using the same procedure, with the exception that D-Glucose was replaced by L-Glucose, which does not react with GOx. This was to ensure that the same mass of glucose was absorbed by each hydrogel sample at the different times and obtained an accurate weight measurement.

The percentage of gel degradation was determined from the following equation:

$$GD = \frac{m_{t_x,D-glucose}}{m_{t_x,L-glucos}} \times 100 \qquad (7)$$

Where, $m_{t_x,D-Glucose}$ is the mass of the freeze-dried hydrogel exposed to D-glucose at time point t_x, $m_{t_x,L-Glucose}$ is the mass of the freeze-dried hydrogel exposed to L-glucose at the same time point t_x.

1.6. Synthesis of RITC-labelled BSA/PNIPAm nanoconjugates

RITC-labelled BSA-NH_2/PNIPAm nanoconjugate was prepared following our previously reported procedure[1]. First, RITC-labelled BSA was synthesized by dissolving in a vial 20.9 mg of BSA in 7.74 mL of Na_2CO_3 buffer (pH 8.5, 100 mM). To this solution 193.5 μL of a DMSO solution of RITC (1.0 mg mL^{-1}) was added. The conjugation reaction was performed for 5 hrs at room temperature. The fluorescent RITC-labelled BSA was purified by dialysis, lyophilized, and stored as a solid at −20 °C. The degree of labelling (DOL) was calculated using UV–Vis spectroscopy [RITC ε(559 nm) = 62,100 L mol^{-1} cm^{-1}; BSA ε(280 nm) = 43,824 L mol^{-1} cm. The dye: protein molar ratio was 1.5:1.

RITC-labelled BSA was then cationized with 1,6-hexanediamine (HMDA). Typically, an aqueous solution of HDMA (500 mg, 4.3 mmol, 5 mL) was adjusted to pH 6.5 using 5 M HCl and added dropwise to a stirred solution of RITC-labelled BSA in MilliQ water (100 mg, 1.5 μmol, 5 mL). The pH was readjusted to 6.5 with 0.1 M HCl. The coupling reaction was initiated by adding 50 mg EDAC to the stirred solution. The pH value was maintained at 6.5 by adding aliquots of 0.1 M HCl, and another 50 mg of EDAC was added after 4h. The solution was stirred overnight at room temperature and finally centrifuged to remove any precipitate (aggregated/crosslinked protein). The supernatant containing the cationized RITC-labelled BSA was then dialyzed for 2 days against Milli-Q water (Medicell dialyzing tubing, MWCO 12-14 kDa), and freeze-dried. This protocol resulted in the attachment of approximately 32 amine groups per BSA molecule, corresponding to a degree of cationization of 32% as measured by MALDI-TOF mass spectrometry.

End-capped mercaptothiazoline-activated PNIPAm (M_n = 7,800 g mol^{-1}) was synthesized following a previously reported protocol.[2] An aqueous solution of the polymer (10 mg in 5 mL) was added dropwise to a stirred solution of cationized RITC-labelled BSA (10 mg in 5 mL of Na_2CO_3 0.1 M, pH = 8.5). The mixed solution was stirred for 12 hrs at 4 °C, then purified by using centrifugal filtration tubes (Millipore, Amicon

Ultra, MWCO 50 kDa) to remove any unreacted PNIPAm and by-products. The resulting BSA/PNIPAm nanoconjugate was characterized by MALDI-TOF MS, which indicated attachment of at least 4 polymer chains per BSA molecule, see **Figure S15**.

Successful synthesis of RITC-labelled BSA-NH_2/PNIPAm nanoconjugate was further confirmed by zeta potential measurements. **Figure S16** shows that the surface charge of BSA passed from +6 mV for native BSA to +35 mV for cationized BSA and eventually to -11 mV for the final BSA/PNIPAm nanoconjugate due to the polymeric coating.

1.7. Preparation of acid-degradable hydrogel-filled proteinosomes

Different proteinosomes samples were prepared with a final concentration of crosslinker (**5**) ranging from 16.7, 83.3, and 167 mg mL^{-1} or 1, 5, and 10 mg by mass. In general, acid-degradable proteinosomes were prepared by mixing in 1.75 mL vials, 15 µL of an aqueous solution of acid-degradable crosslinker (**5**) in Na_2CO_3 buffer solution (pH 8.5, 0.1 M) with 30 µL of an aqueous solution of RITC-labelled BSA/PNIPAm (8.0 mg mL^{-1}), and an aqueous solution of CM-chitosan (15 µL, 40 mg mL^{-1}, pH 8.5-9). Mixing was immediately followed by the addition of 1 mL of 2-ethyl-1-hexanol. The mixture was shaken by hand for 60 s. Samples were prepared at a constant aqueous/oil volume fraction (φ_w) of 0.06. The acid-degradable crosslinker (**5**) was allowed to react with the primary amine groups of CM-chitosan and the interfacial primary amine groups of the BSA/PNIPAm nanoconjugates for 48 hrs. After 48 hrs, the clear upper oil layer was discarded, and 1 mL of a 70% ethanol/Na_2CO_3 buffer (pH=8.5, 10 mM) mixture was used to dissolve the emulsion. The solution was then dialyzed against 70%, 50%, and 30% ethanol/Na_2CO_3 buffer (pH=8.5, 10 mM) for 2 hrs, then against carbonate buffer for 1 day to complete the transfer of the crosslinked proteinosomes into an alkaline solution. Proteinosomes were then let to sediment at the bottom of the vial overnight at 4 °C. The day after, the supernatant was removed and replaced in 1 mL of Na_2CO_3 buffer (pH 8.5, 10 mM). Proteinosomes remained stable over several days if stored in Na_2CO_3 and kept at 4 °C.

pH-stable proteinosome samples for control experiments were prepared following the same protocol described above with the exception that acid-degradable crosslinker (**5**) was replaced by pH-stable crosslinker PEG-diNHS (M_n 2000 Da) with a final concentration in the aqueous phase of 33.3 mg mL^{-1} or 2 mg by mass, and the proteinosomes were transferred using ethanol/water mixtures.

GOx-containing proteinosomes were prepared following the same general procedure described above with the only exception that 15 µL of a solution of GOx (50 mg mL^{-1}) in Na_2CO_3 (pH 8.5, 0.1 M) were added to the water phase containing the BSA/PNIPAm nanoconjugate, the CM-chitosan and the acid-degradable crosslinker (**5**).

Proteinosomes containing an FITC-labelled CM-chitosan hydrogel were prepared following the same general procedure with the exception that 15 µL of a 1:2 mixture of FITC-labelled CM-chitosan and unlabeled CM-chitosan was used instead of only unlabeled CM-chitosan. The FITC-labelled CM-chitosan was prepared by adding 2 mL of a solution of FITC in DMSO (1.0 mg mL^{-1}) to 10 mL of a solution CM-chitosan (20 mg mL^{-1}) in Na_2CO_3 buffer (0.1 M, pH=8.5). The reaction was stirred overnight, and the tagged polymer was dialyzed against MillQ water for 1 day. FITC-labelled CM-chitosan was then freeze-dried and redissolved in Na_2CO_3 buffer (0.1 M, pH=8.5) to make a stock solution of polymer, at the concentration of 40 mg ml^{-1}.

1.8. Time-dependent fluorescence microscopy studies of acid-mediated proteinosome degradation

On a glass microscope slide 50 µL of HCl 1.0 M was added to 150 µL of proteinosome sample. The sample was monitored on a manual inverted fluorescence microscope at ×20 magnification. Time-lapse images were acquired at time intervals of 1 min. Images were processed using Image j software.

Control experiments were carried out using the same protocol with the exception that acid-sensitive proteinosomes crosslinked with crosslinker (**5**) were replaced with acid-stable proteinosomes crosslinked with commercially available PEG-diNHS.

1.9. Time-dependent FACS analysis of acid-mediated proteinosome degradation

Aliquots of water dispersed pH-sensitive proteinosomes crosslinked with acid-degradable crosslinker (**5**) (150 µL) were incubated with 1 M HCl (50 µL) for defined time intervals varying from 0 to 2.5 hrs. Aliquots of the incubated samples (150 µL) were transferred into a test tube and analyzed with a Fortessa X-20 flow cytometer (BD Biosciences) for 45 seconds at low pressure with a 100 µm sorting nozzle. Recorded data were processed with FlowJo software version 10.0.7 (Tree Star).

Aliquots of water dispersed pH-sensitive GOx-containing proteinosomes were incubated in a 0.5 M aqueous solution of D-glucose (50 µL) for defined time intervals varying from 0 to 32 hrs. Aliquots of the incubated samples (150 µL) were transferred into a test tube and analyzed with a Fortessa X-20 flow cytometer (BD Biosciences) for 45 seconds at low pressure with a 100 µm sorting nozzle. Recorded data were processed with FlowJo software version 10.0.7 (Tree Star).

2. Supplementary Figures

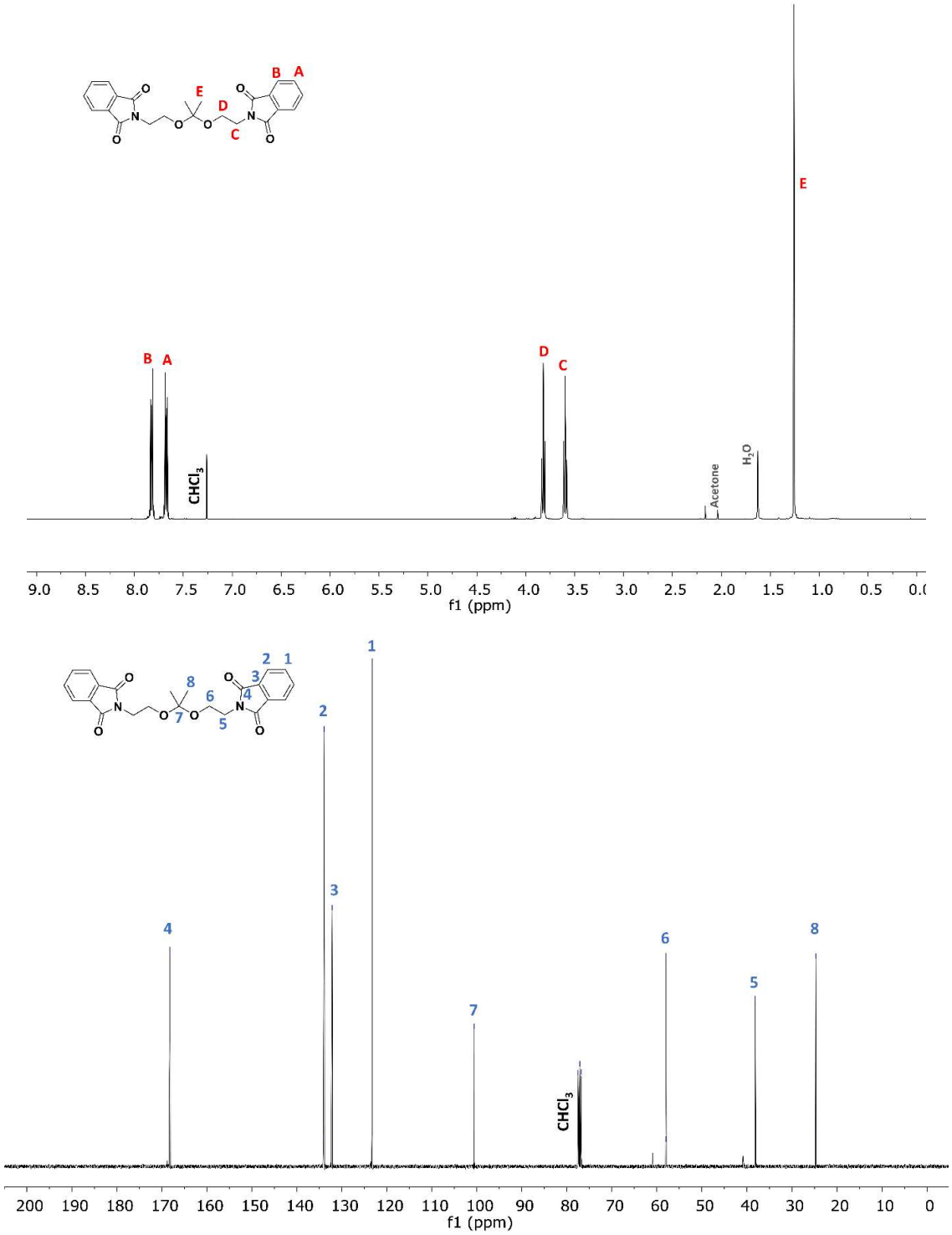


Figure S1: ^{1}H (top) and ^{13}C NMR (bottom) spectra of 2,2-bis(phthalimidoethoxy)propane (**1**) acquired in $CDCl_3$. Spectra were obtained using a Varian 400 MHz spectrometer. Residual solvent peaks have been labelled in the ^{1}H NMR spectrum.

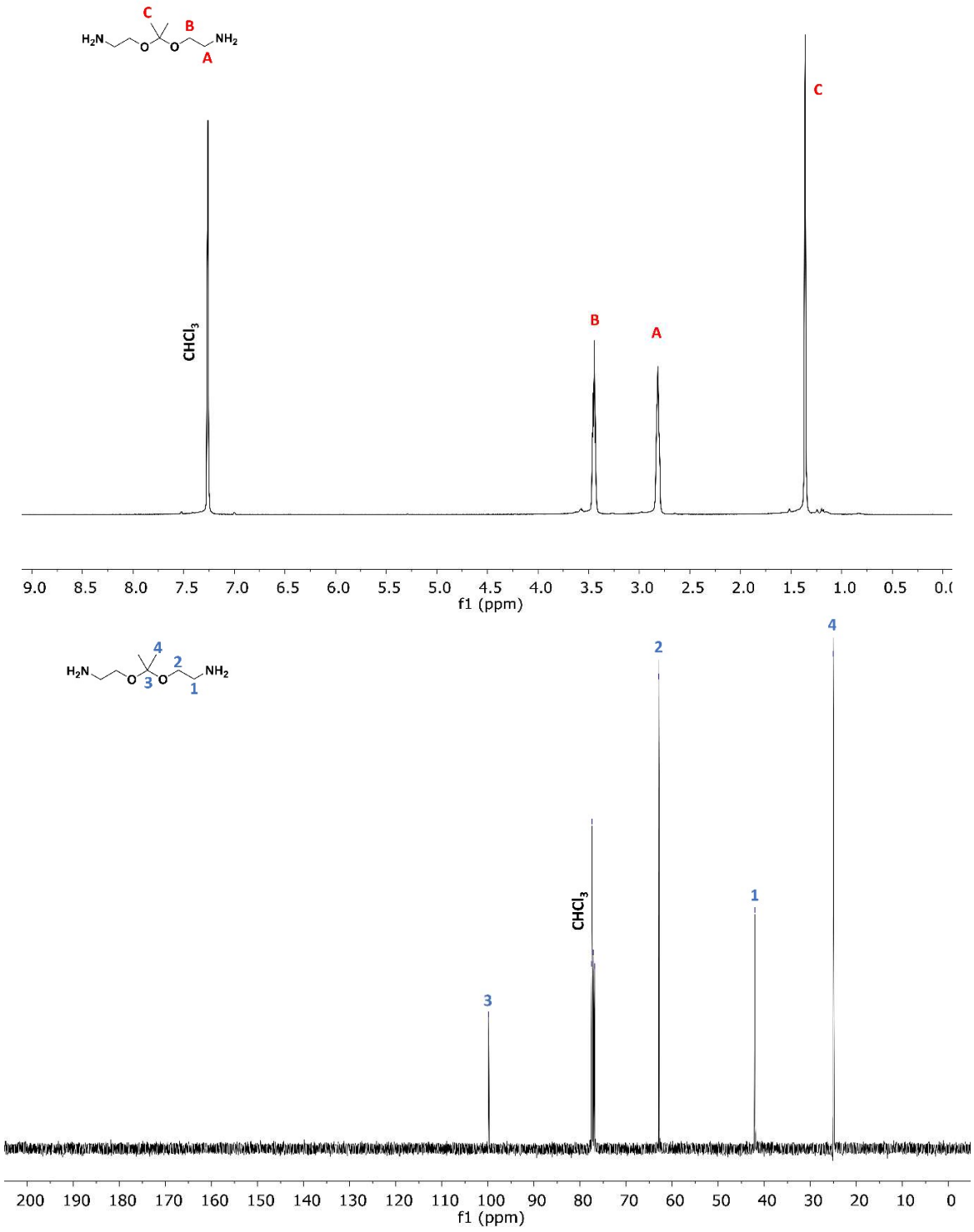


Figure S2: ^{1}H (top) and ^{13}C NMR (bottom) spectra of 2,2-bis(aminoethoxy)propane (**2**) acquired in $CDCl_3$. Spectra were obtained using a Varian 400 MHz spectrometer. Residual solvent peaks have been labelled in the ^{1}H NMR spectrum.

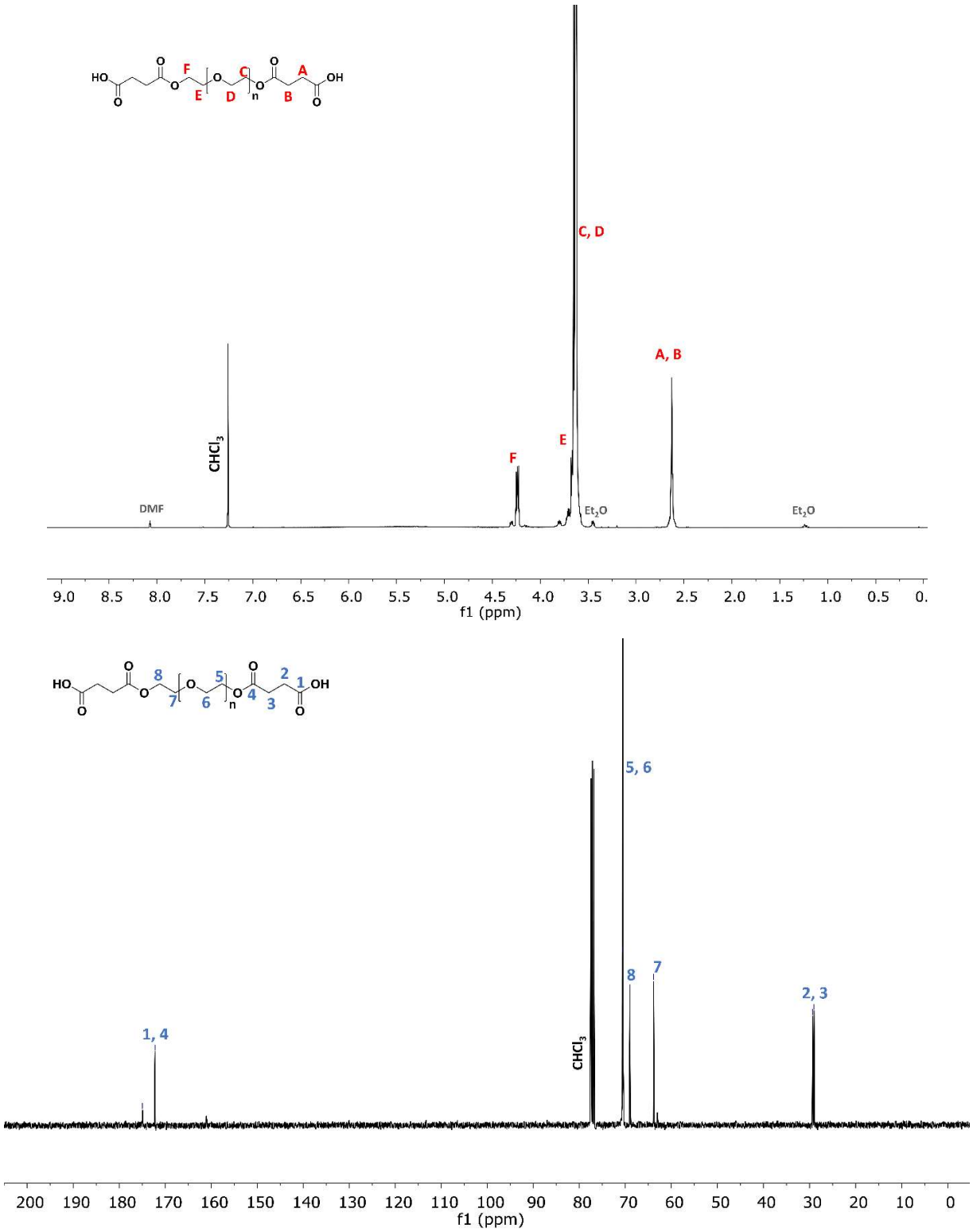


Figure S3: ^{1}H (top) and ^{13}C NMR (bottom) spectra of bis(succinic acid ester)polyethylene glycol (**3**) acquired in $CDCl_3$. Spectra were obtained using a Varian 400 MHz spectrometer. Residual solvent peaks have been labelled in the ^{1}H NMR spectrum.

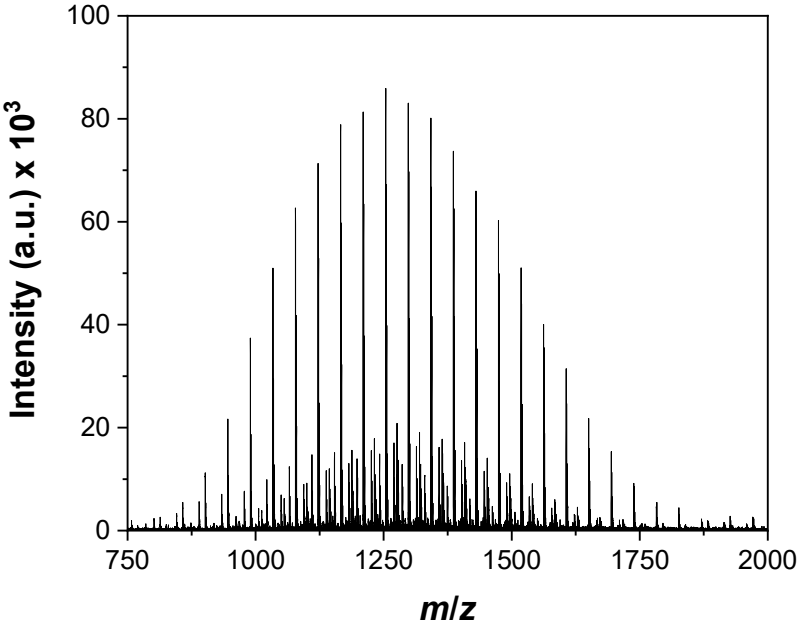


Figure S4: MALDI-TOF mass spectrum of bis(succinic acid ester)polyethylene glycol (**3**)*,* M_n = 1250.

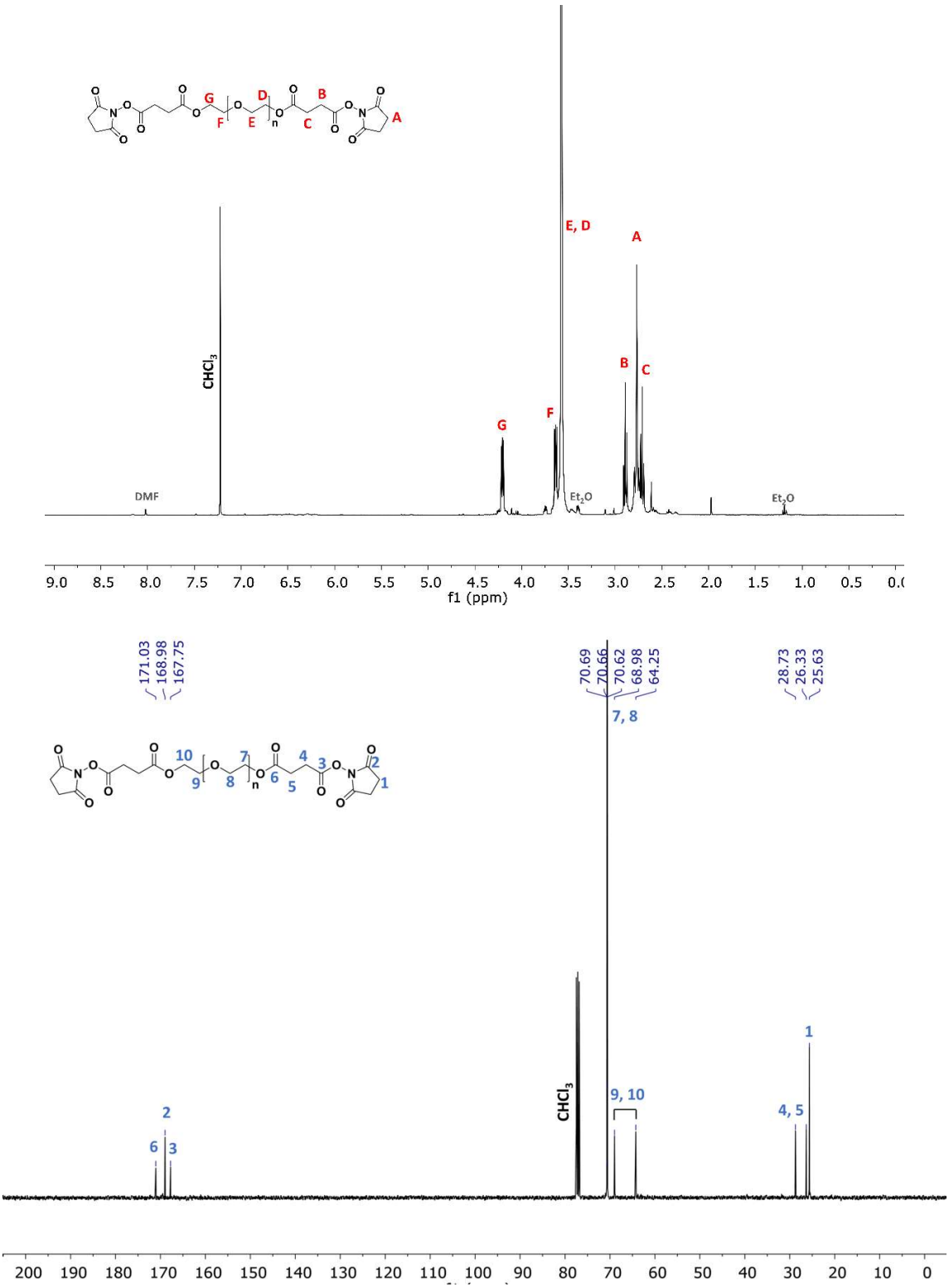


Figure S5: ^{1}H (top) and ^{13}C NMR (bottom) spectra of *O*,*O*′-bis[2-(*N*-succinimidyl-succinate]polyethylene glycol (**4**) acquired in $CDCl_3$. Spectra were obtained using a Varian 400 MHz spectrometer. Residual solvent peaks have been labelled in the ^{1}H NMR spectrum.

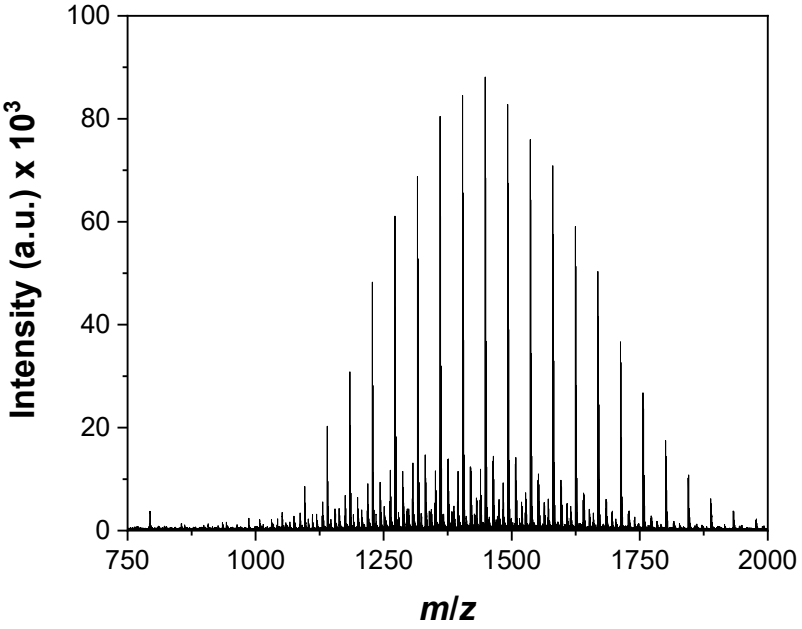


Figure S6: MALDI-TOF mass spectrum of O,O'-bis[2-(N-succinimidyl-succinate]polyethylene glycol (**4**), M_n = 1400 Da.

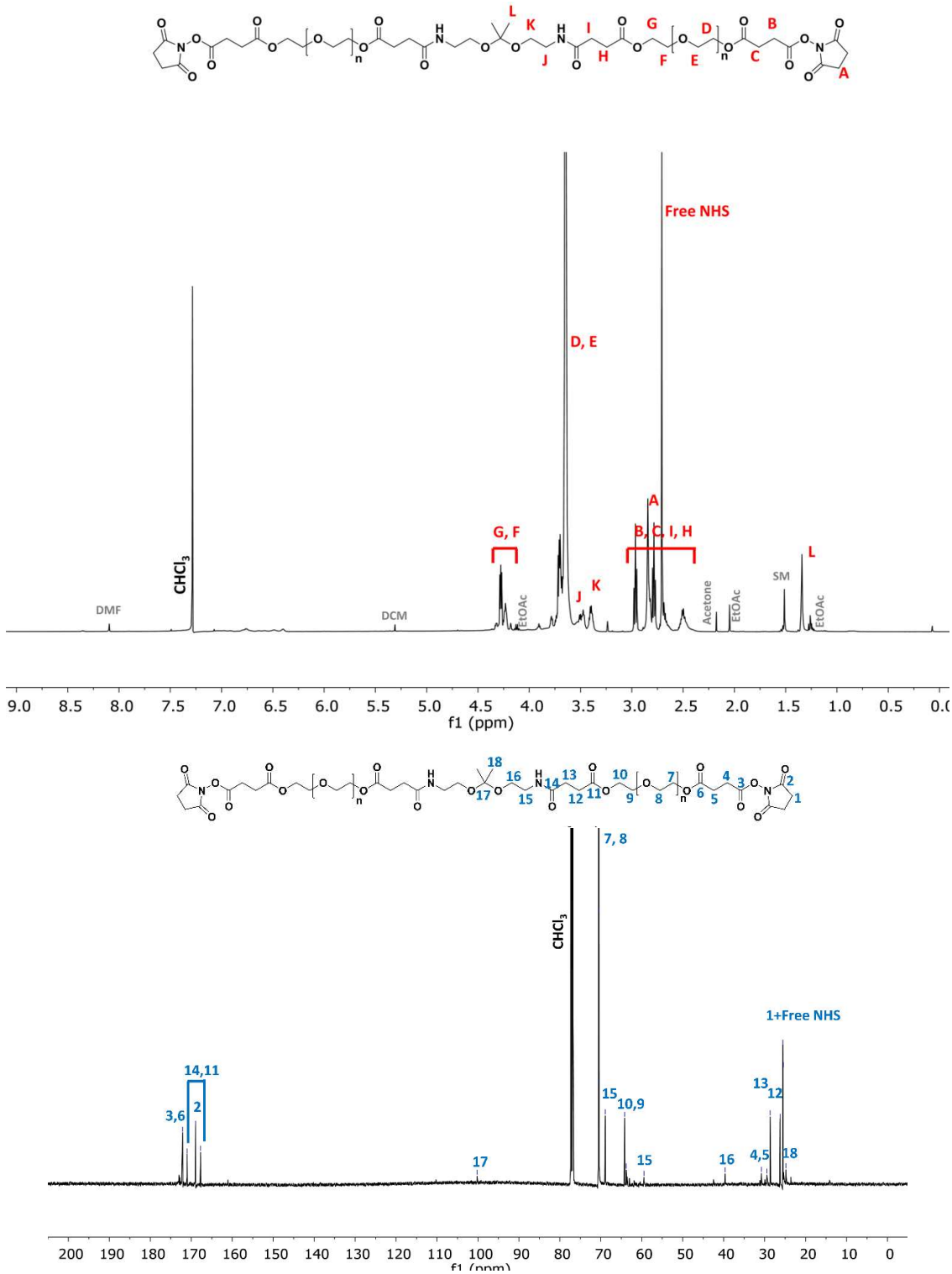


Figure S7: ^{1}H (top) and ^{13}C NMR (bottom) spectra of 2,2-bis({*O*-[(*N*-succinimidyl)succinyl]-*O*'-[succinylamino]polyethylene glycolethoxy)propane (**5**) acquired in $CDCl_3$. Spectra were obtained using a Varian 400 MHz spectrometer. Residual solvent peaks have been labelled in the ^{1}H NMR spectrum. The spectrum shows also presence of ketal and NHS ester hydrolysis products.

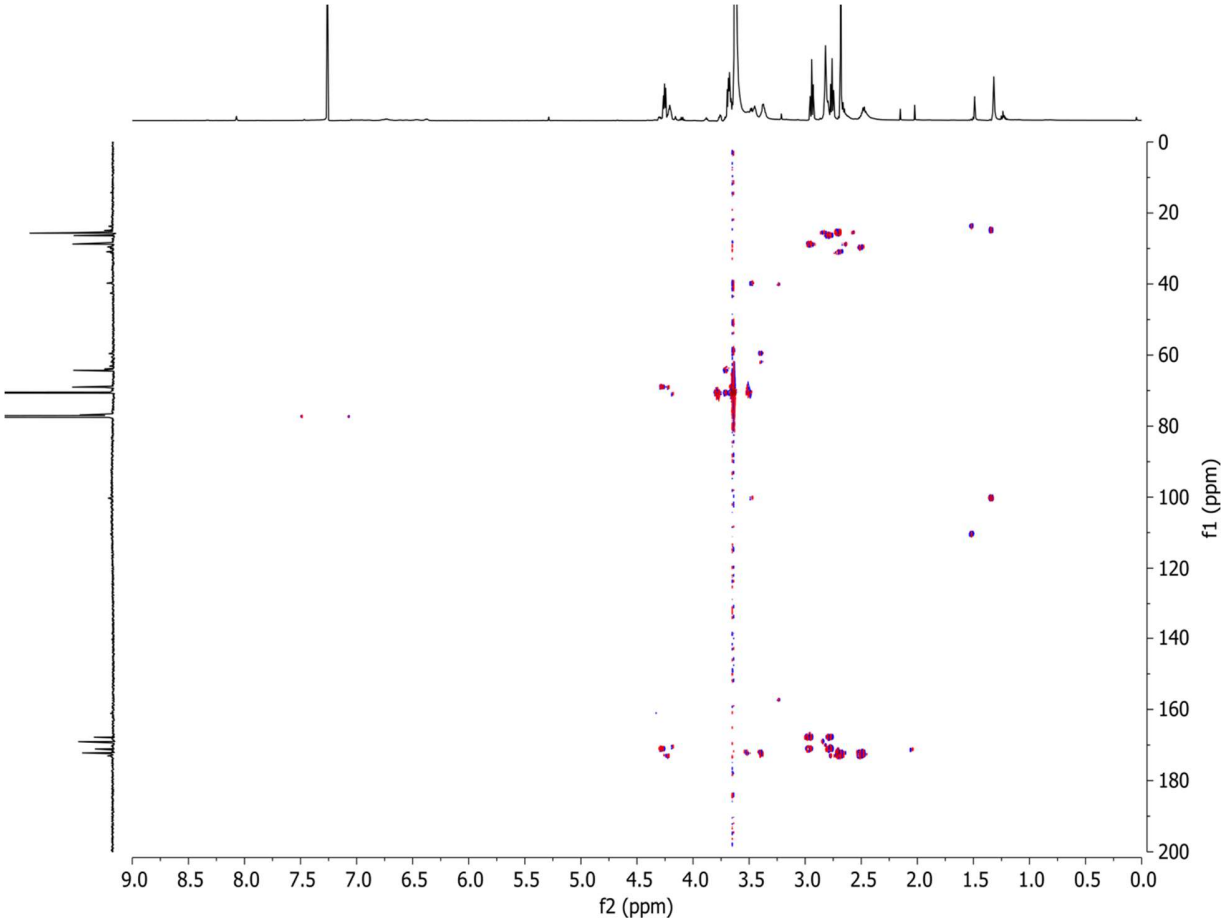


Figure S8: HMBC spectra of 2,2-bis({*O*-[(*N*-succinimidyl)succinyl]-*O*'-[succinylamino]polyethylene glycol}ethoxy)propane (**5**) acquired in $CDCl_3$. Spectra were obtained using a Varian 500 MHz spectrometer. The HMBC was used to correctly assign all the proton and carbon peaks of compound (**5**) as shown in **Figure S7**.

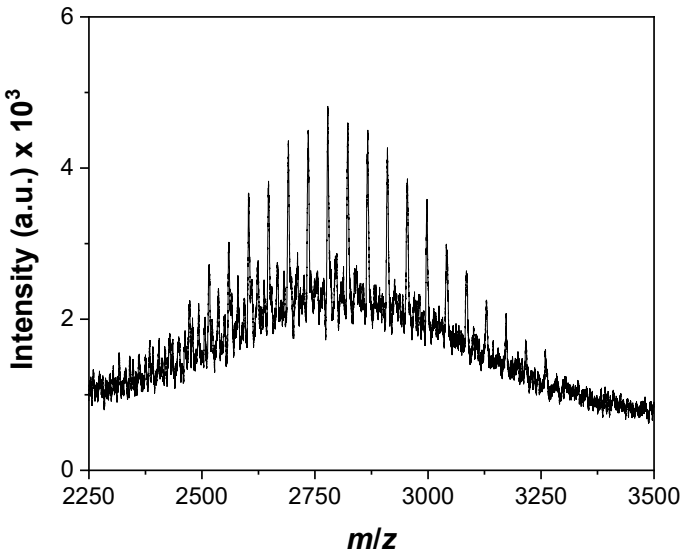


Figure S9: MALDI-TOF mass spectrum of acid-cleavable crosslinker (**5**), M_n = 2800 Da.

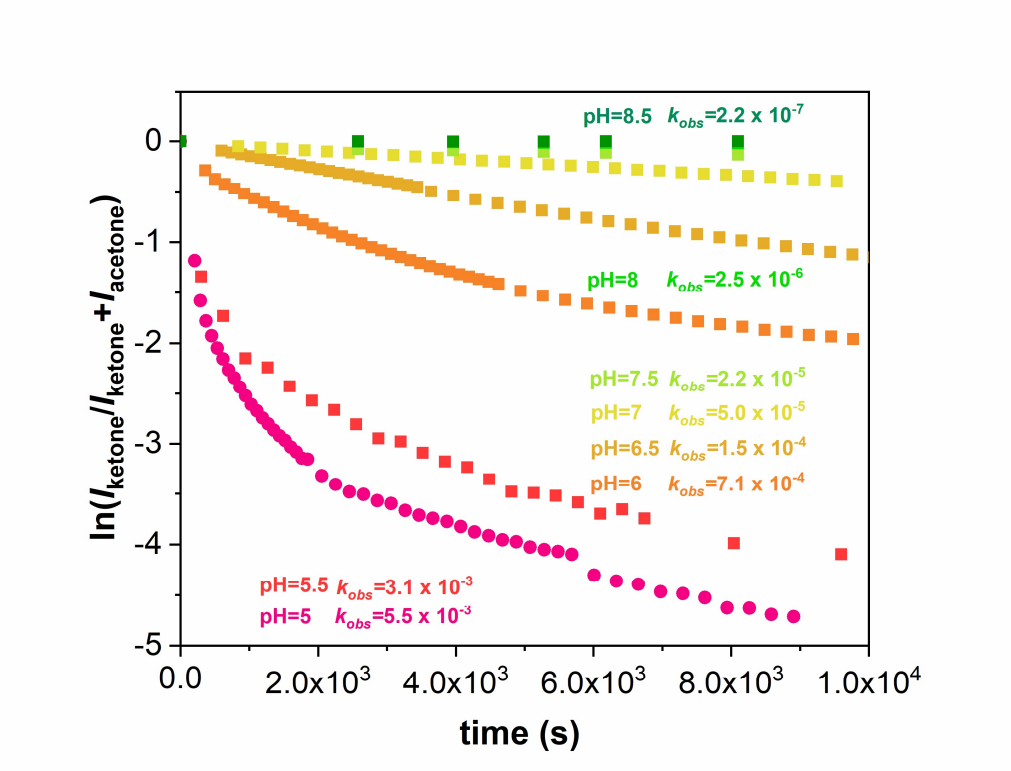


Figure S10 Plots of $\ln\left(\frac{I_{ketal}}{I_{acetone}+I_{ketal}}\right)$ against incubation time for the acid catalyzed hydrolysis of crosslinker (**5**) at different pH. K_{obs} was determined as a negative slope of the initial reaction rate (see **Supplementary Section 1.3**).

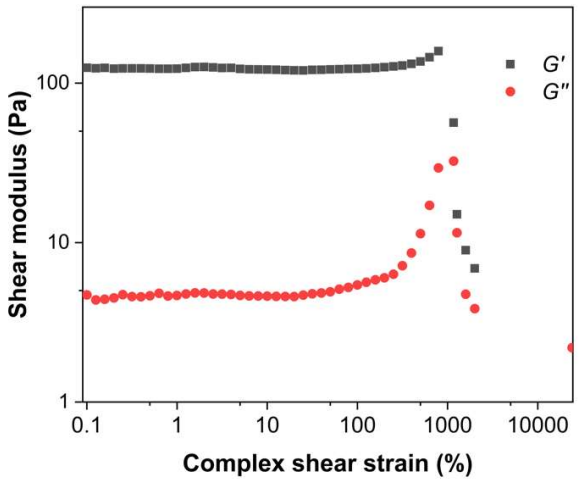


Figure S11. Strain sweeps from 0.01 to 2,000% strain carried out at a frequency of ω_0 = 1.0 Hz (6.3 rad s^{-1}) on a freshly made hydrogel sample prepared by mixing 1.5 mL of a solution of CM-chitosan 40 mg mL^{-1} with 15 mg of acid-degradable crosslinker. Both the elastic (G') and viscous (G'') components of the shear modulus remained linear in the region from 1 to 250% strain. The graph is reported in logarithmic scale.

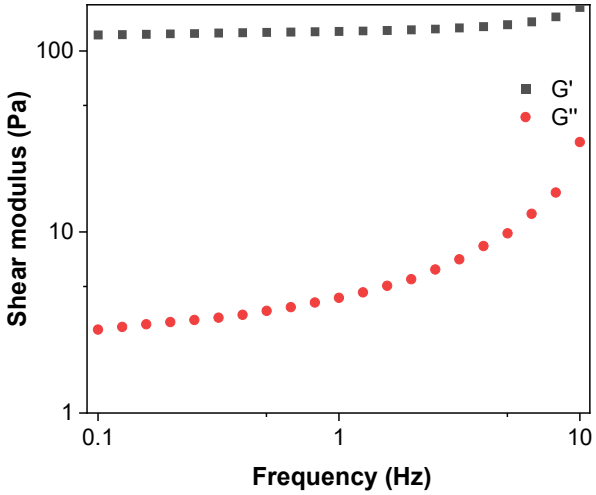


Figure S12. Frequency sweeps from 0.01 to 10 Hz carried out at a set strain of 10% on a freshly made hydrogel sample prepared by mixing 1.5 mL of a solution of CM-chitosan 40 mg mL^{-1} with 15 mg of acid-degradable crosslinker. The graph is reported in logarithmic scale.

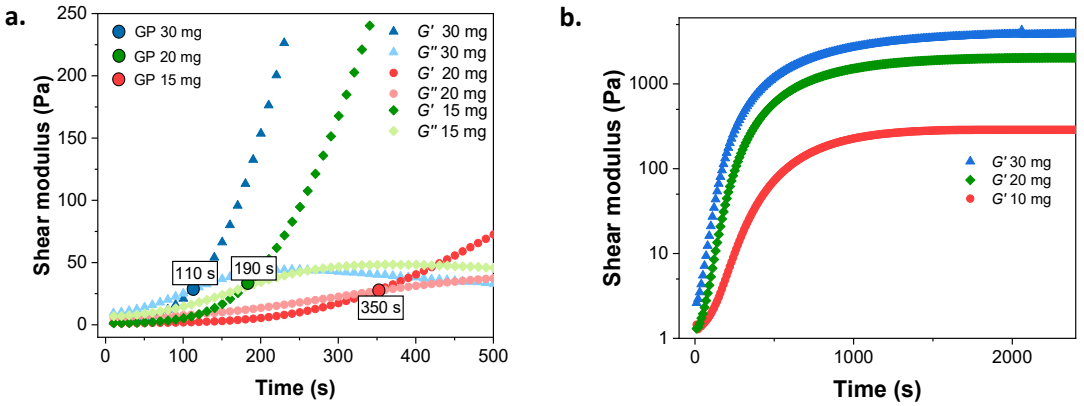


Figure S13. a. Time-dependent changes of elastic (*G'*) and viscous modulus (*G''*) of the hydrogels in (**a**). The crossover point of *G*' and *G*'' for each hydrogel sample has been highlighted in the graph. These points indicate the sol-gel transition times (gelation point, GP). **b.** Time-dependent changes in the elastic modulus (*G'*, logarithmic scale) of CM-chitosan hydrogels crosslinked with 10, 20 and 30 mg of crosslinker (**5**). For all samples *G'* reached a plateau after *ca.* 1000 s.

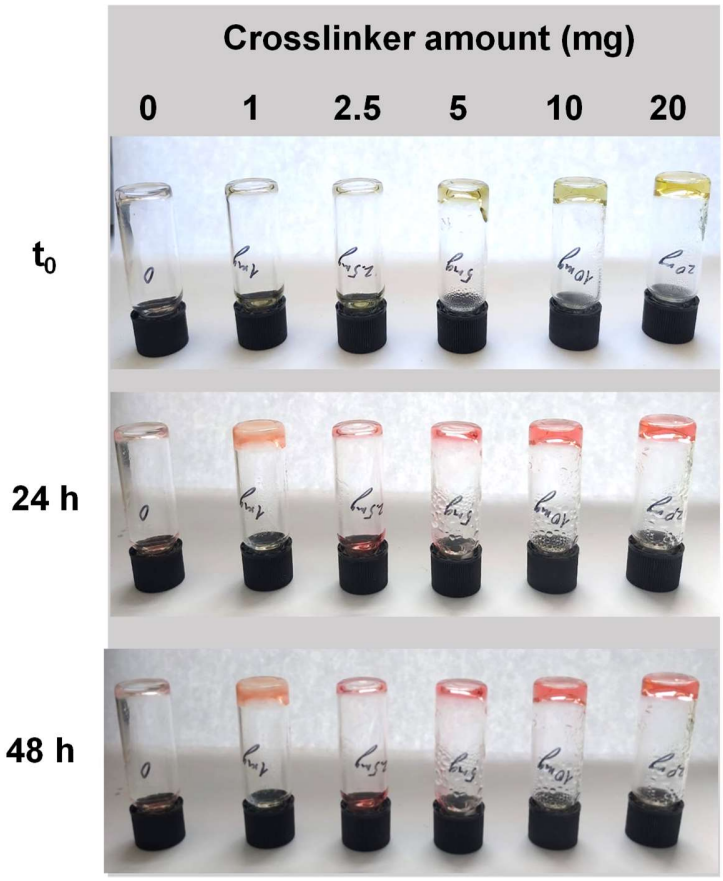


Figure S14. Time-dependent photographs of inverted vials containing CM-chitosan hydrogels (4 wt%, 200 µL) crosslinked with different amounts of pH-stable PEG-diNHS crosslinker ranging from 0 to 20 mg, as specified on the top of the figure. The top photograph (t_0) shows the presence of the hydrogel at the beginning of the experiment. The hydrogels were then exposed to 5 µL of HCl (1 M) and photographs were acquired after 1, 24, and 48 hrs as indicated on the left-hand side of the figure. The hydrogels were preloaded with a universal indicator, which showed an alkaline pH at t_0 (top row, yellow colour) and acidic pH (pink colour) after addition of HCl. Due to the use of the pH-stable PEG-diNHS crosslinker instead of the acid-degradable crosslinker (**5**) all hydrogel did not decompose over time.

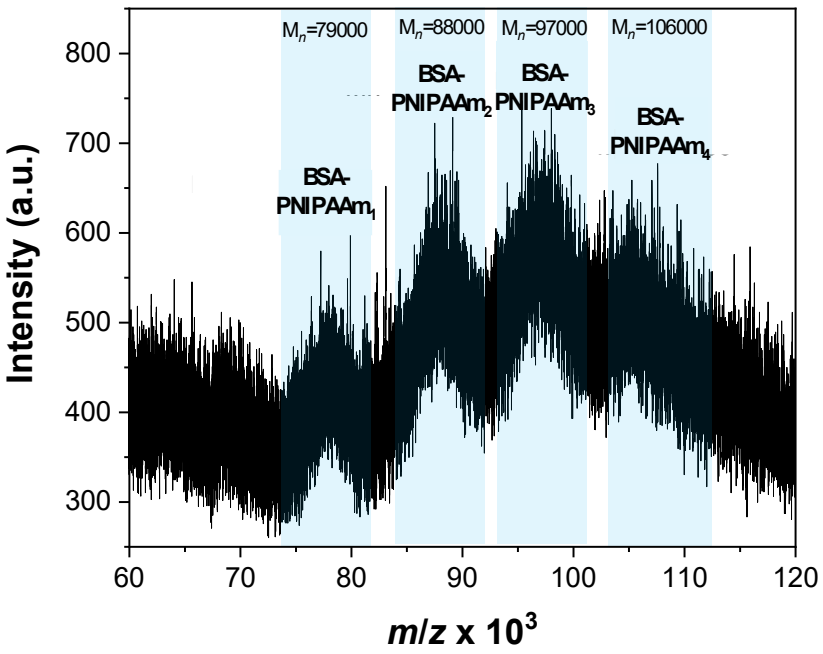


Figure S15. MALDI-TOF mass spectrum of a sample of RITC-labelled BSA-NH_2/PNIPAm nanoconjugate. For convenience, the different types of protein-polymer nanoconjugate generated by conjugating a mercaptothiazoline-activated PNIPAm (M_n 9,000 g mol^{-1}) onto cationized BSA are highlighted in the graph.

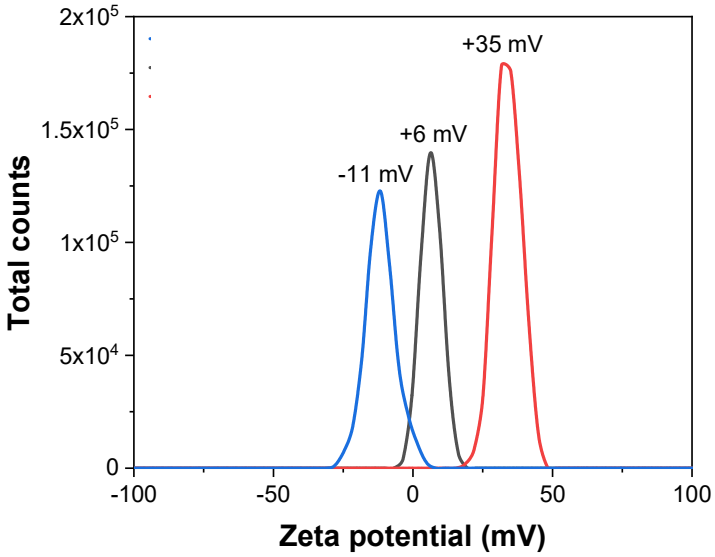


Figure S16. Zeta potential measurements for BSA (black plot), cationized BSA (red plot) and BSA/PNIPAm nanoconjugate (blue plot) acquired at a concentration of 1 mg mL^{-1} in pre-filtered MilliQ water and at room temperature.

3. Supplementary Videos

Video S1: Fluorescence microscopy video (λ_{ex} = 530 nm) showing the slow disassembly of RITC-labelled acid-sensitive hydrogel-filled proteinosomes in the presence of HCl 250 mM. Progressive hydrolysis of the ketal crosslinker resulted in the progressive proteinosome dissolution associated with a time-dependent decrease in the proteinosomes' red fluorescence intensity. Scale bar = 50 μm.

4. Supplementary References

[1] Bin Liu and S. Thayumanavan, *J. Am. Chem. Soc*. **2017**, 139, 2306–2317
[2] X. Huang, M. Li, D. C. Green, D. S. Williams, A. J. Patil, S. J. N. c. Mann, *Nat. Comm.*, **2013**, *4*, 1–29.