Electronic Supplementary Material (ESI) for Polymer Chemistry.

Cationic glycopolymers through controlled polymerisation of a glucosamine-based monomer mimicking the behaviour of chitosan

ELECTRONIC SUPPLIMENTARY INFORMATION

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Materials

All materials were reagent grade and used as received, unless otherwise specified: acetic acid (glacial, Chem Supply), 1,1'-azobis(cyclohexanecarbontilrile) (ACCN, 98%, Sigma Aldrich), dimethylformamide (DMF, Ajax Finechem), 1,4-dioxane (>99%, Sigma-Aldrich), boron trifluoride diethyl etherate (synthesis grade, Sigma Aldrich), butyl acrylate (BA, 99%, Sigma Aldrich), chitosan LMW (Sigma Aldrich), chloroform (Merck), cyclohexane (Chem Supply), dichloromethane (DCM, Merck), ethyl acetate (Chem Supply), ethyl trifluoroacetate (99%, Sigma Aldrich), fluorescein isothiocyanate (FITC, > 90%, Sigma Aldrich), D-(+)-glucosamine hydrochloride (>99%, Sigma Aldrich), 2-hydroxyethyl acrylate (HEA, 96%, Sigma Aldrich), methanol (>98%, Ajax Finechem), *N,N*-dimethylacetamide (DMAc, >99.9%, Sigma-Aldrich), pentafluoro styrene (PFS, 99%, Sigma Aldrich), phthalic anhydride (99%, Sigma Aldrich), pyridine (99.8%, Sigma Aldrich), sodium bicarbonate ($NaHCO_3$, Chem Supply), sodium carbonate (Na_2CO_3, Chem Supply) and sodium methoxide (NaOMe, 25wt%, Sigma Aldrich), triethylamine (TEA, 99%, Sigma Aldrich). The RAFT agents, 3-benzylsulfanylthiocarbonylsufanylpropionic acid (BSPA) and 4-cyanopentanoic acid dithiobenzoate (CPADB), were synthesised as previously[1, 2]. The initiator, 2,2'-Azobisisobutyronitrile (AIBN) was re-crystallized twice from methanol. MilliQ was produced internally and had a resistivity of 18.2 mΩ/cm.

Instrumental Analysis

Nuclear magnetic resonance (^{1}H-NMR)

All NMR analysis was conducted on a Bruker Avance III HD instrument operating at either 400 MHz or 600MHz. The instrument and deuterated solvent is assigned for each spectrum within the caption. All spectra were collected using a minimum of 8 scans and all chemical shifts are reported in ppm (δ) relative to TMS.

DMF gel permeation chromatography (GPC)

The GPC system for polymer characterisation was a Shimadzu system consisting of a CBM-20A system controller, DGU-20A3 online degasser, LC-20AD pump (1 mL/min), SIL-20AHT auto-injector (50 μL), RID-20A refractive index detector (290nm) and CTO-20A column over (50 °C). Three Phenomenex columns (10^5, 10^4 and 10^3 Å pore size, 5 μm particle size) and one Phenogel Linear guard column (5 μm) were used for analysis. The mobile phase was DMF containing 0.1% w/v LiBr and 0.004% w/v BHT. Commercial polystyrene and poly(methyl methacrylate) standards were used for calibration, where the range of molecular weights of the standards were 100 – 10^5 g/mol. Samples were dissolved in DMF (ca. 4 – 5 mg/mL) and passed through a 0.45 μm filter prior to injection. Chromatographs were processed using LabSolutions Postrun Analysis Software.

Dynamic light scattering (DLS)

DLS measurements were conducted on a Bookhaven ZetaPlus Particle Sizer with a Dust-Cutoff value of 40. For size determination, polymer samples were prepared in MilliQ water at a concentration of 0.5 – 1 mg/mL and then filtered through a 0.45 μm filter to remove any dust particulates just before sample measurement. Each sample was measured 5 times and averaged to give the listed values.

Transmission electron microscopy (TEM)

TEM images were obtained with a JEOL1400 instrument where the working beam voltage was 80-100 kV. Samples were prepared by placing a drop of relevant micelle variant solution on a FORMVAR-coated copper grid (400 mesh). The solution was left for 30 min, under cover, to allow settling of particles on to the grid. Excess solution was removed using filter paper and the grids were air dried before being stained with uranyl acetate (2 % aqueous solution) for 2 min. Excess stain was removed and grids air dried before observation.

Laser scanning confocal microscopy

Tight junction visualisation was observed under a Zeiss LSM 780 laser scanning confocal microscope system. The system equipped with a DPSS 561-10 laser connected to a Zeiss Axio Observer.Z1 inverted microscope. The ZEN2012 imaging software (Zeiss) was used for image acquisition and processing. The monolayer was observed with a 20 × 0.8 NA oil objective.

Fluorescence measurement

Fluorescence intensity of all samples was measured on a Cary Eclipse Fluorescence Spectrophotometer. Instrument parameters were the same for all samples, where excitation and emission slit width was set at 5 nm. For samples containing FITC (chitosan, P(GluHEA) and M-30-F), the excitation and wavelength was 499 nm and emission wavelength was 520 nm. For Lucifer Yellow,

excitation wavelength was 428 nm and emission wavelength was 530 nm. All measurements were zeroed against HBSS/HEPE buffer solution. Where required, samples were diluted in HBSS/HEPES buffer (typical dilution factor was 1 : 9). All fluorescence measurements were conducted in triplicate where each sample contained two parallel studies. The fluorescence data is represented as mean ± standard deviation, where n = 2. A two-way analysis of variance (ANOVA) was used to analyse statistical difference between groups. Tukey's post hoc test was used for multiple comparisons to highlight any difference between groups.

Synthesis and characterisation

Synthesis of 1-acryloylethyl-2-(trifluoroacetamido)-3,4,6-Tri-*O*-acetyl-α,β-D-glucopyranoside (GluCF$_3$HEA)

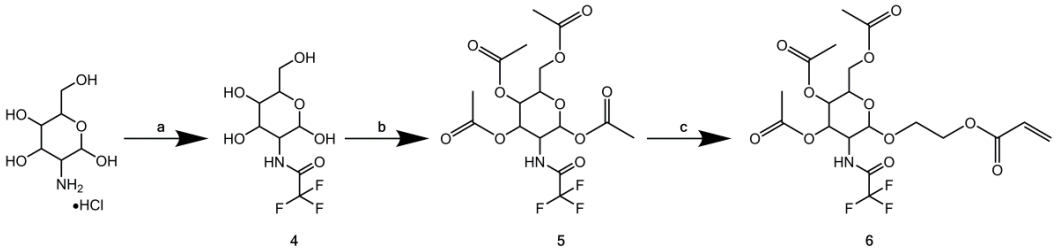

Figure S1: Overall scheme for synthesis of GluCF$_3$HEA monomer where *(a)* Na_2CO_3, ethyl trifluoroacetate, 24 h, RT; *(b)* acetic anhydride, pyridine, 48 h, RT; *(c)* boron trifluoride diethyl etherate, 2-hydroxyethyl acrylate, 48 h, RT.

2-(trifluoroacetamido)-α,β-D-glucopyranoside (**4**) was synthesised according to literature[3]. Briefly, glucosamine hydrochloride (9.99 g, 4.63 × 10^{-2} mol) was added to two-necked flask fitted with a stirring bar. The flask was sealed and purged with nitrogen for 5 min before methanol (anhy., 100 mL) was added and the mixture stirred at room temperature for 30 min. After 30 min, the reaction was placed under nitrogen again and Na_2CO_3 (10.02g, 9.45 × 10^{-2} mol) quickly added to the flask before it was resealed and placed under continuous nitrogen flow for 20 min. Ethyl trifluoroacetate (13.5 mL, 1.14 × 10^{-1} mol) was added dropwise under stirring and nitrogen before the mixture sealed and left to stir for 24 h at room temperature. After 24 h, the mixture was transferred to a roundbottom flask and the solvent removed under reduced pressure to afford an off-white slurry. The slurry was resuspended in ethyl acetate : methanol : water (10 : 2 : 0.2, v/v/v) and loaded onto a silica column (40 – 63 μm) running the same eluent. The desired product eluted as two spots (α, β form, R_f = 0.5 – 0.6) and the solvent removed under reduced pressure to afford an off-white, but clear gel. The gel was redissolved in methanol and the solvent removed again to afford a white powder.

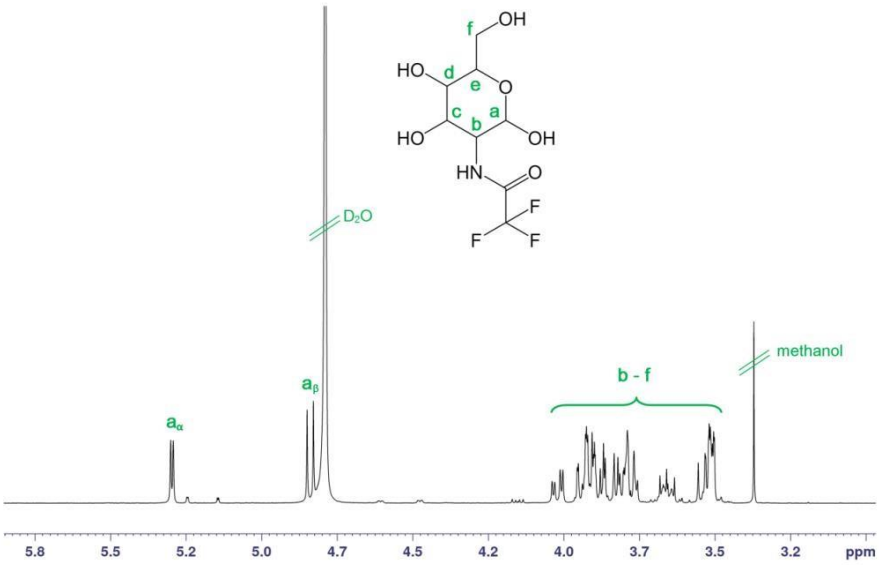

Figure S2: ^{1}H-NMR spectra and assignment of 2-(trifluoroacetamido)-α,β-D-glucopyranoside (**1**) in D_2O and at 400 MHz.

1,3,4,6-Tetra-*O*-acetyl-2-(trifluoroacetamido)-α,β-D-glucopyranoside (**5**) was synthesised according to literature[4]. Briefly, **4** (10.20g, 3.27 × 10^{-2} mol) was added to a two-necked flask fitted with a stirring bar and dropping funnel before the vessel was sealed and purged with nitrogen for 5 min. Pyridine (anhy., 75 mL) was added to the flask and the mixture stirred for 10 min before acetic anhydride (31.6 mL, 3.34 × 10^{-1} mol) was added dropwise. The mixture was left under a continuous stream of nitrogen for 30 min before the vessel was sealed and left to stir at room temperature for 48 h. After 48 h, the dark brown solution was poured into cold water (80 mL) and extracted with DCM (80 mL, 3 ×). The organic phases were combined and washed successively with 150 mL of cold water (2 ×), 3% HCl (6 ×), water and saturated NaCl. The organic phase was dried over sodium sulfate and removed under reduced pressure to afford a thick yellow liquid. The product was recrystallised from diethyl ether as white crystals.

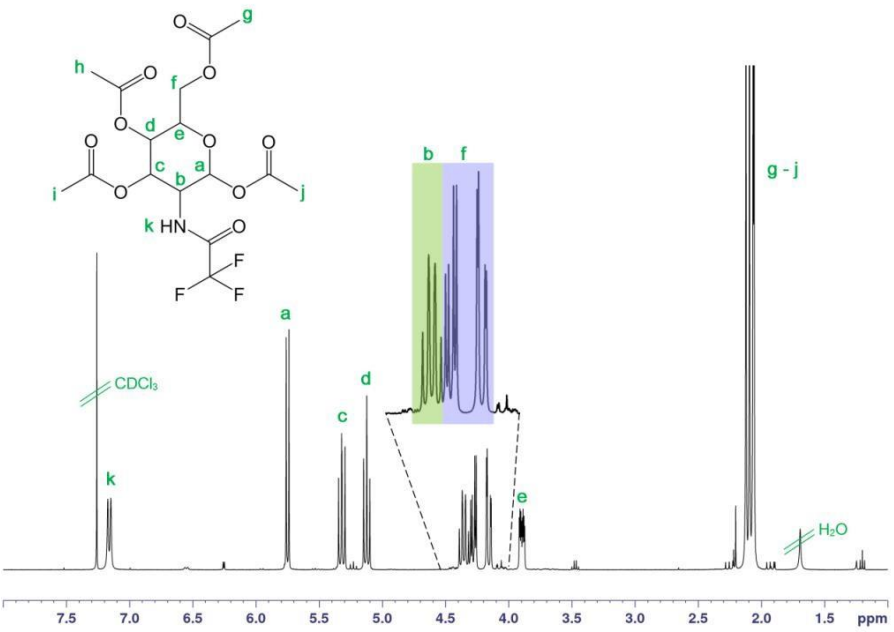

Figure S3: ^{1}H-NMR spectra and assignment of 1,3,4,6-Tetra-O-acetyl-2-(trifluoroacetamido)-α,β-D-glucopyranoside (**2**) in $CDCl_3$ and at 400 MHz.

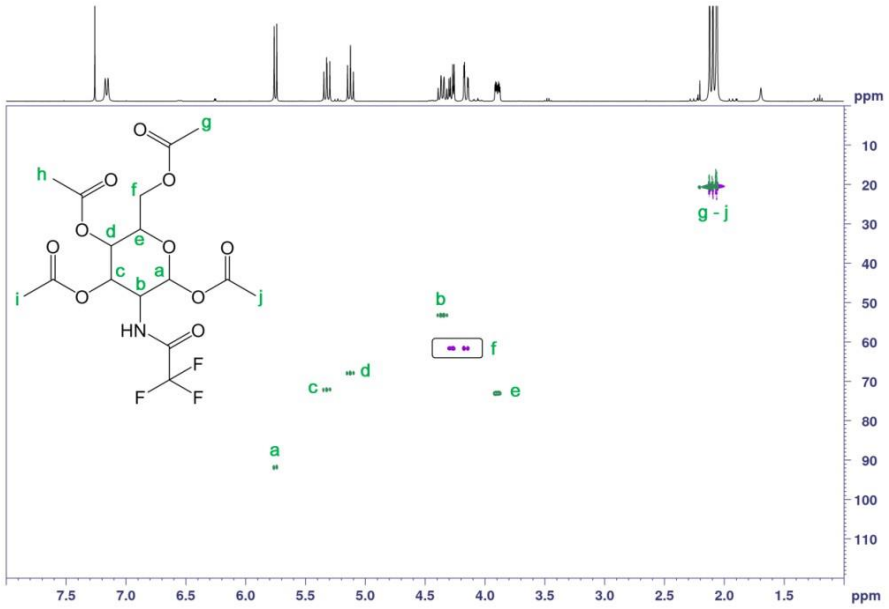

Figure S4: ^{1}H – ^{13}C (HSQC) 2D NMR spectra and assignment of 1,3,4,6-Tetra-O-acetyl-2-(trifluoroacetamido)-α,β-D-glucopyranoside (**2**) in $CDCl_3$ and at 400 MHz.

5 (6.28 g, 1.42 × 10^{-2} mol) was added to a two-necked flask fitted with 3Å molecular sieves and a dropping funnel. The flask was purged with nitrogen for 5 min before DCM (anhy., 100 mL) was transferred into the flask. DCM (anhy., 20 mL) was also transferred into the dropping funnel. 2-hydroxylethyl acrylate (3.25 mL, 2.83 × 10^{-2} mol) was added to the flask and boron trifluoride diethyl etherate (8.80 mL, 7.08 × 10^{-2} mol) was added to the dropping funnel. 10 min after DCM addition to **5**, boron trifluoride diethyl etherate was added dropwise to the reaction flask over the course of 1 h.

The reaction was left under a continuous stream of nitrogen for 30 min before the flask was sealed and left in an orbital shaker at room temperature for 48 h. The crude reaction mixture was extracted against cold water (100 mL) and the aqueous phase further backwashed with DCM (2 × 50 mL). The organic phases were combined and washed with 100 mL of water (3 ×), saturated NaCl and water. The organic phase was dried over sodium sulfate and the solvent was removed under reduced pressure to give light brown oil. The monomer **6** was purified via two flash chromatography columns. The first column ran ethyl acetate : cyclohexane (1 : 1, v/v) to separate the protected sugar **2** (R_f = 0.41) from the glucosamine monomer **6** and HEA (R_f = 0.33). The solvent was removed under reduced pressure to give a white solid. The solid was dissolved in the eluent of the second column, which was composed of ethyl acetate : cyclohexane (2 : 1, v/v) to separate the glucosamine monomer **6** (R_f = 0.63) from unreacted HEA (R_f = 0.48). After removal of the solvent under reduced pressure, **6** was obtained as white crystals (40 % yield).

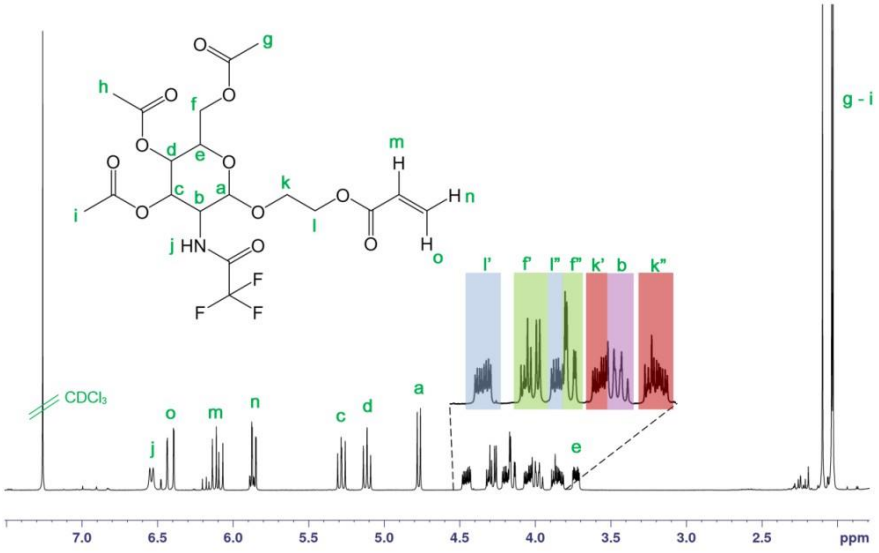

Figure S5: ^{1}H-NMR spectra and assignment of 1-acryloylethyl-2-(trifluoroacetamido)-3,4,6-Tri-O-acetyl-α,β-D-glucopyranoside (GluCF_3HEA) in $CDCl_3$ and at 400 MHz.

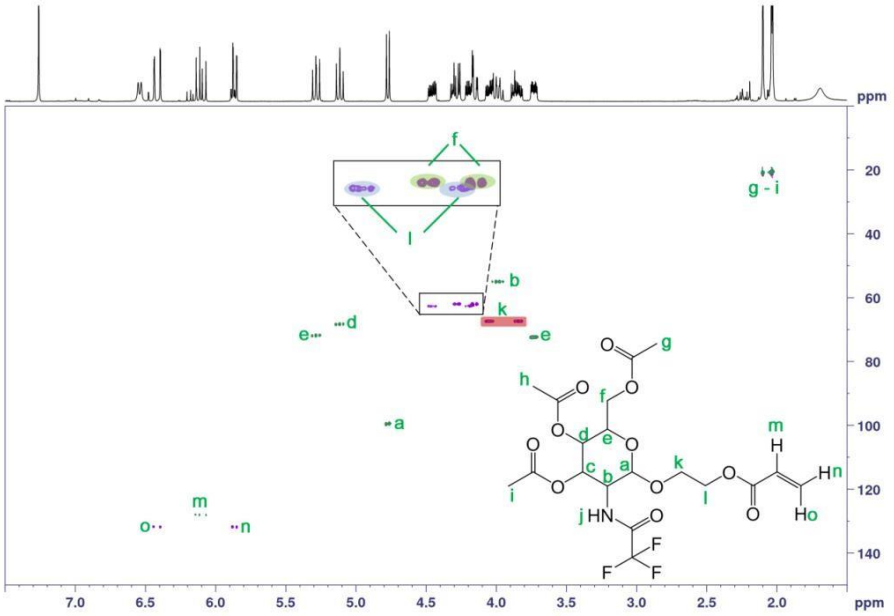

Figure S6: ^{1}H – ^{13}C (HSQC) 2D NMR spectra and assignment 1-acryloylethyl-2-(trifluoroacetamido)-3,4,6-Tri-O-acetyl-α,β-D-glucopyranoside ($GluCF_3HEA$) in $CDCl_3$ and at 400 MHz

Synthesis of 1-acryloylethyl-2-(isoindoline-1,3-dione)-3,4,6-Tri-*O*-acetyl-β-D-glucopyranoside (GluPAHEA)

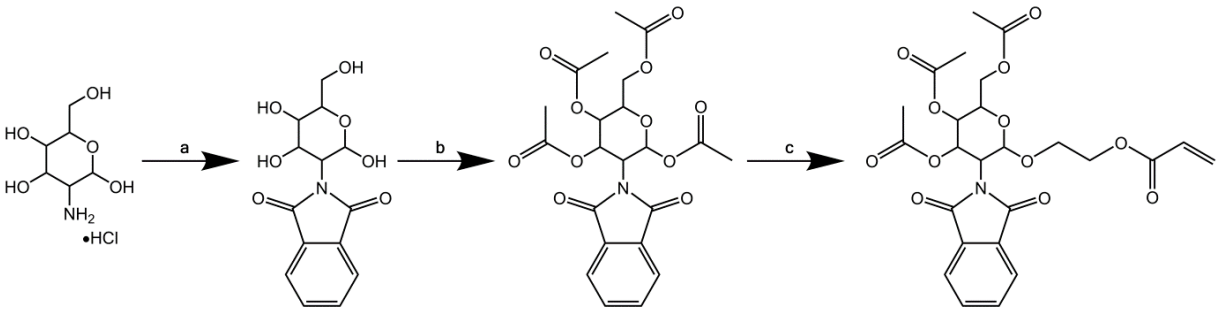

Figure S7: Overall scheme for the synthesis of GluPAHEA monomer where *(a)* NaOMe, TEA, phthalic anhydride, 24 h, RT; *(b)* acetic anhydride, pyridine, 24 h, RT; *(c)* boron trifluoride diethyl etherate, 2-hydroxyethyl acrylate, 48 h, RT.

The synthesis of 1,3,4,6-Tetra-*O*-acetyl-2-(isoindoline-1,3-dione)-D-glucopyranoside was prepared as previously described[5] The general procedure is outlined here. 2-(isoindoline-1,3-dione)-β-D-glucopyranoside was synthesized batchwise by initially dissolving D-glucosamine hydrochloride (5.00 g, 2.32×10^{-2} mol) in methanol (18.0 mL). With stirring, sodium methoxide (25 wt% in methanol, 3.71×10^{-2} mol, 8.50 mL) was added slowly and the resultant mixture was stirred vigorously for 15 min. The white precipitate was removed via filtration and washed with methanol to give a bright yellow liquid. Triethylamine (2.55×10^{-2} mol, 3.55 mL) and half of the phthalic anhydride were added and the mixture stirred vigorously for 15 min. The remaining phthalic anhydride was added (total 3.78 g, 2.55×10^{-2} mol) and the mixture stirred for a further 15 min at room temperature and then at 50°C

for 20min. Small volumes of methanol was added as required when the viscosity of the mixture became too thick. The mixture was cooled in an icebath for 1 hr before the solids were collected by filtration, washed with methanol and dried under vacuum to afford 2-(isoindoline-1,3-dione)-D-glucopyranoside (**1**) as an off-white powder (4.82 g, 67%). The ^{1}H-NMR spectrum of 2-(isoindoline-1,3-dione)-β-D-glucopyranoside is shown in Figure S8.

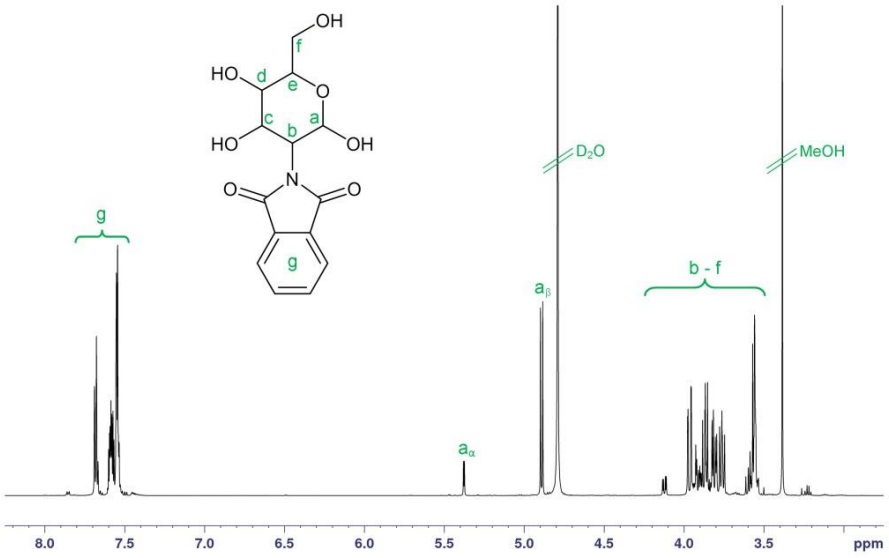

Figure S8: ^{1}H-NMR of 2-(isoindoline-1,3-dione)-β-D-glucopyranoside (compound **1**) in D_2O at 400MHz.

2-(isoindoline-1,3-dione)-β-D-glucopyranoside (27.0 g, 8.73 × 10^{-2} mol) was suspended in anhydrous pyridine (100 mL) under nitrogen. Acetic anhydride (62.1 mL, 3.44 × 10^{-1} mol) was added dropwise to the reaction mixture before the system was sealed and allowed to react at RT for 24 hr with vigorous stirring. The resultant brown solution was poured into cold water (150 mL) and extracted with chloroform (2 × 300 mL). The organic phase was combined and extracted sequentially with 250 mL of cold water (2 ×), 3% HCl (7 ×), saturated $NaHCO_3$ (2 ×) and cold water (1 ×). The organic phase was then dried over sodium sulfate and chloroform removed under reduced pressure to afford a dark yellow liquid.

The yellow liquid was dissolved in the minimum amount of diethyl ether at room temperature. The solvent was then concentrated under reduced pressure at 40°C and the product recrystallised overnight to give an off-white powder as crude 1,3,4,6-Tetra-*O*-acetyl-2-(isoindoline-1,3-dione)-D-glucopyranoside. The product was again recrystallised from ethanol and then ethyl acetate to afford the beta-glucopyranoside product, 1,3,4,6-Tetra-*O*-acetyl-2-(isoindoline-1,3-dione)-β-D-glucopyranoside, as white crystals (**2**, 9.22 g, 62%). The ^{1}H-NMR and ^{1}H-^{13}C (HSQC) 2D spectra of 1,3,4,6-Tetra-*O*-acetyl-2-(isoindoline-1,3-dione)-β-D-glucopyranoside is shown in Figure S9 and Figure S10, respectively.

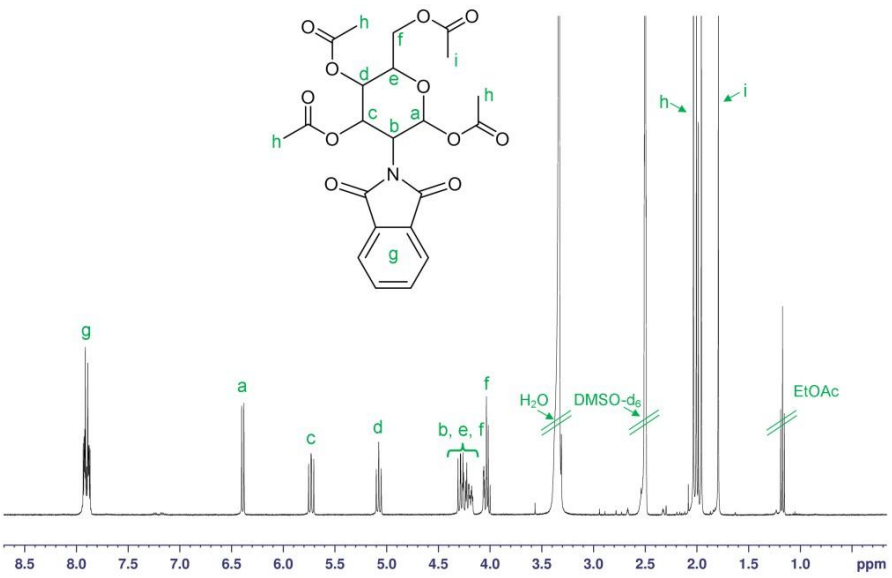

Figure S9: ^{1}H-NMR of 1,3,4,6-Tetra-*O*-acetyl-2-(isoindoline-1,3-dione)-D-glucopyranoside (compound **2**) in DMSO-d_6 at 400MHz.

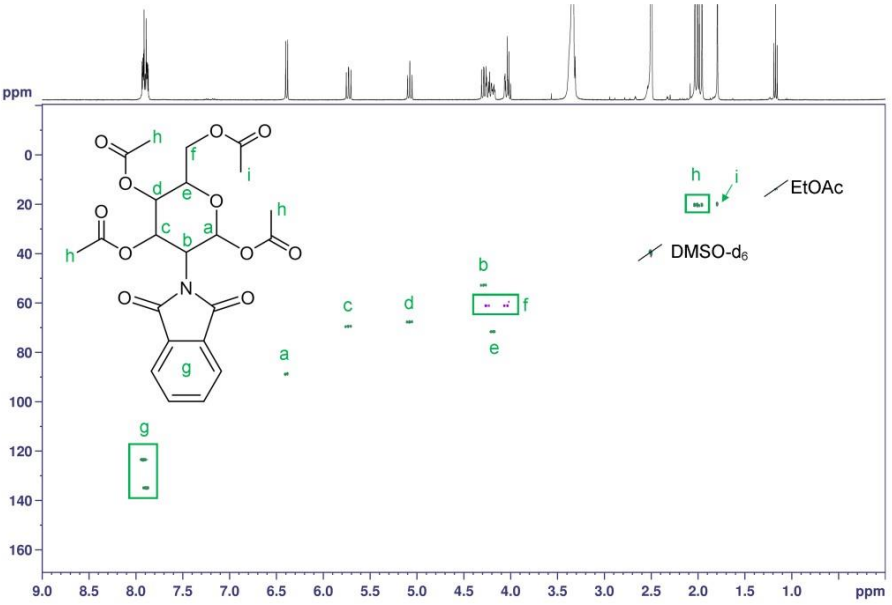

Figure S10: ^{1}H – ^{13}C (HSQC) 2D NMR spectra and assignment of 1,3,4,6-Tetra-*O*-acetyl-2-(isoindoline-1,3-dione)-D-glucopyranoside (compound 2) in DMSO-d_6 and at 400 MHz.

1,3,4,6-Tetra-*O*-acetyl-2-(isoindoline-1,3-dione)-β-D-glucopyranoside (10.0 g, 2.09 × 10^{-2} mol), 2-hydroxethyl acrylate (4.86 g, 4.19 × 10^{-2} mol) and molecular sieves (4Å, 5 g) were added to a flask and the system purged with nitrogen for 10 min. Under nitrogen, anhydrous DCM (80 mL) was added to the flask and the reagents allowed to dissolve before BF_3OEt_2 (13.0 mL in 30 mL anhydrous DCM, 1.05 × 10^{-1} mol) was added dropwise to the reaction mixture with occasional shaking of the flask. After a further 5 min of nitrogen flow, the flask was sealed and reacted for 48 hr in an orbital shaker (120 rpm). The resulting brown solution was poured into cold water (60 mL) and extracted

with 60 mL of DCM (1 ×) and saturated $NaHCO_3$ (1 ×), respectively, before the organic phase was dried over sodium sulfate. DCM was removed under reduced pressure to afford a viscous yellow liquid as crude 1-acryloylethyl-2-(isoindoline-1,3-dione)-3,4,6-Tri-*O*-acetyl-β-D-glucopyranoside.

The crude solution was dry-loaded onto silica (40 – 63 μm) at *ca.* 27 wt% using DCM before the solvent was removed under reduced pressure. The monomer was purified via silica gel chromatography (toluene/EtOAc 2:1, R_f = 0.42) to give the product as a pale, off-white solid upon solvent removal under reduced pressure. 1-acryloylethyl-2-(isoindoline-1,3-dione)-3,4,6-Tri-*O*-acetyl-β-D-glucopyranoside was obtained at 98% purity with the other 2% being unreacted 1,3,4,6-Tetra-*O*-acetyl-2-(isoindoline-1,3-dione)-β-D-glucopyranoside (8.12 g, 73%). The ^{1}H-NMR and ^{1}H-^{13}C (HSQC) 2D spectra of 1,3,4,6-Tetra-*O*-acetyl-2-(isoindoline-1,3-dione)-β-D-glucopyranoside is shown in Figure S11 and Figure S12, respectively.

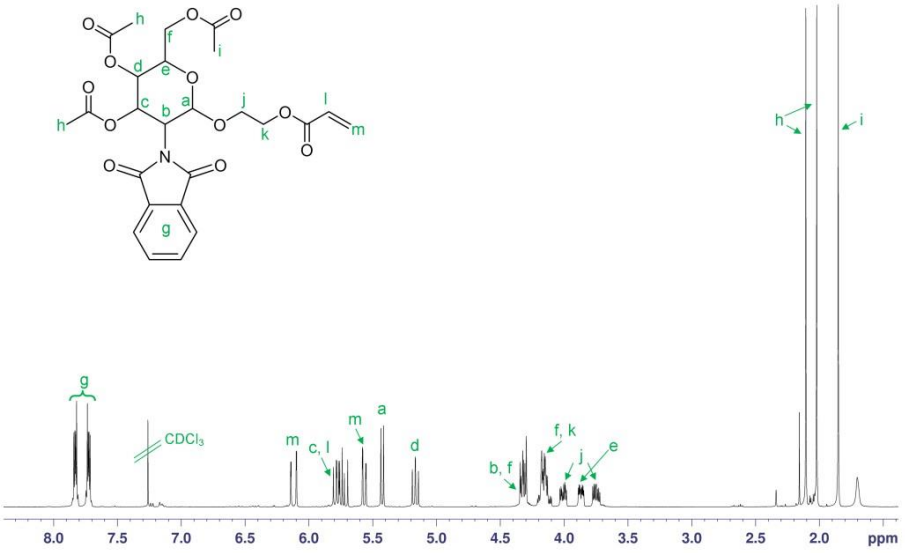

Figure S11: ^{1}H-NMR of 1-acryloylethyl-2-(isoindoline-1,3-dione)-3,4,6-Tri-*O*-acetyl-β-D-glucopyranoside (compound **3**, GluPAHEA) in $CDCl_3$ at 400MHz.

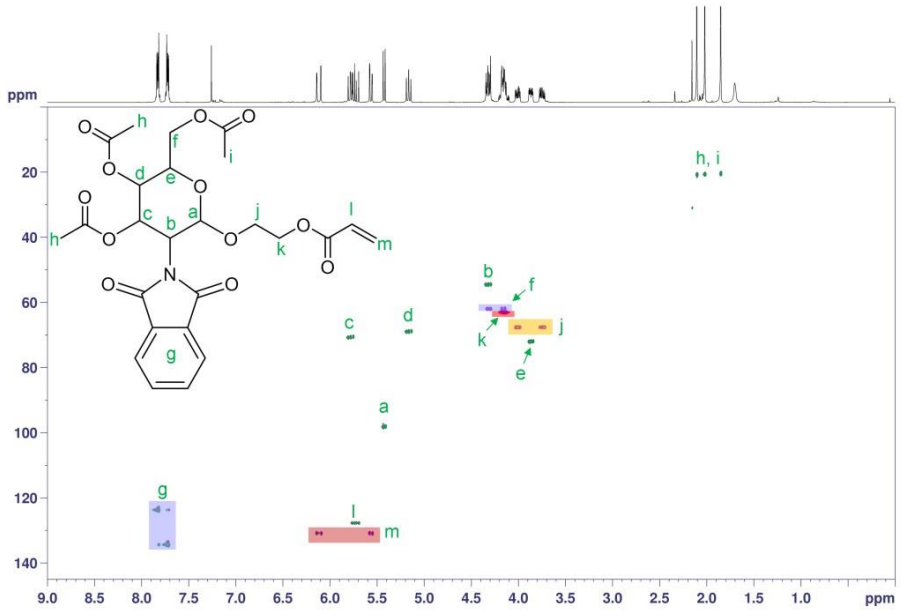

Figure S12: ^{1}H – ^{13}C (HSQC) 2D NMR spectra and assignment of 1,3,4,6-Tetra-*O*-acetyl-2-(isoindoline-1,3-dione)-D-glucopyranoside (compound **3**, GluPAHEA) in DMSO-d_6 and at 400 MHz.

Kinetics Study of the polymerisation of GluPAHEA

GluPAHEA (500.0 mg, 1.13×10^{-3} mol) was dissolved in 500 µL of toluene. ACCN (108 µL of 10 mg/mL solution, 4.42×10^{-6} mol) and CPADB (309 µL of 20 mg/mL solution, 2.21×10^{-5} mol) was added to the monomer solution and thoroughly mixed. The reaction mixture was divided into five separate vials, sealed and all vials degassed with nitrogen gas for 15 min. The polymerisations were allowed to proceed at 85°C and the first sample removed after 100 min. The polymerisation was quenched by simultaneously immersing the reaction into an icebath and exposing the reaction to air. Further samples were removed at regular intervals afterwards. The stacked GPC traces of the polymers recovered at various timepoints is shown in Figure S13 and Figure S14 depicts *Đ* values for the polymers as well as radical concentration throughout the study with time. Table S1 lists the characteristics of each polymer. The ^{1}H-NMR spectrum and assignment of relevant peaks of the purified homopolymer is shown in Figure S15.

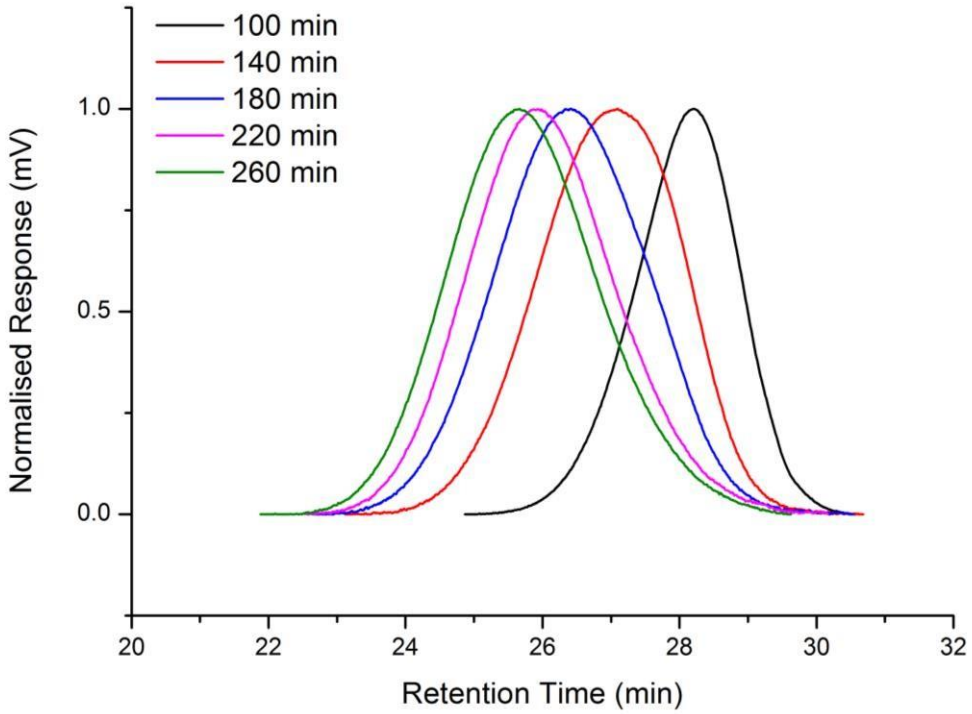

Figure S13: Stacked GPC traces of P(GluPAHEA) at various timepoints in the kinetics study of GluPAHEA polymerisation

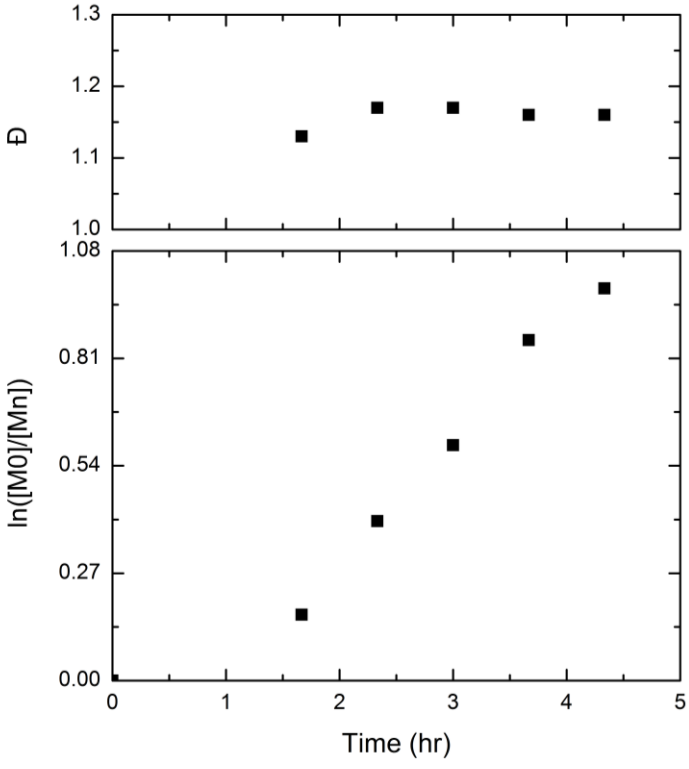

Figure S14: *(Top) p*olydispersity of P(GluPAHEA) at various timepoints in the kinetics study of GluPAHEA polymerisation and *(bottom)* radical concentration with time during the same kinetics study

Table S1: Characteristics of P(GluPAHEA) obtained at various timepoints in the kinetics study of GluPAHEA polymerisation

Time Point (min)	Conversion (%)[a]	Theo. Mn[a] (g/mol)	Mn[b] (g/mol)	Đ[b]
100	15	4 300	4 200	1.13
140	33	9 100	6 700	1.17
180	45	12 100	8 300	1.17
220	58	15 600	9 600	1.16
260	63	17 000	10 500	1.16

[a]Calculated by NMR, [b]calculated by GPC

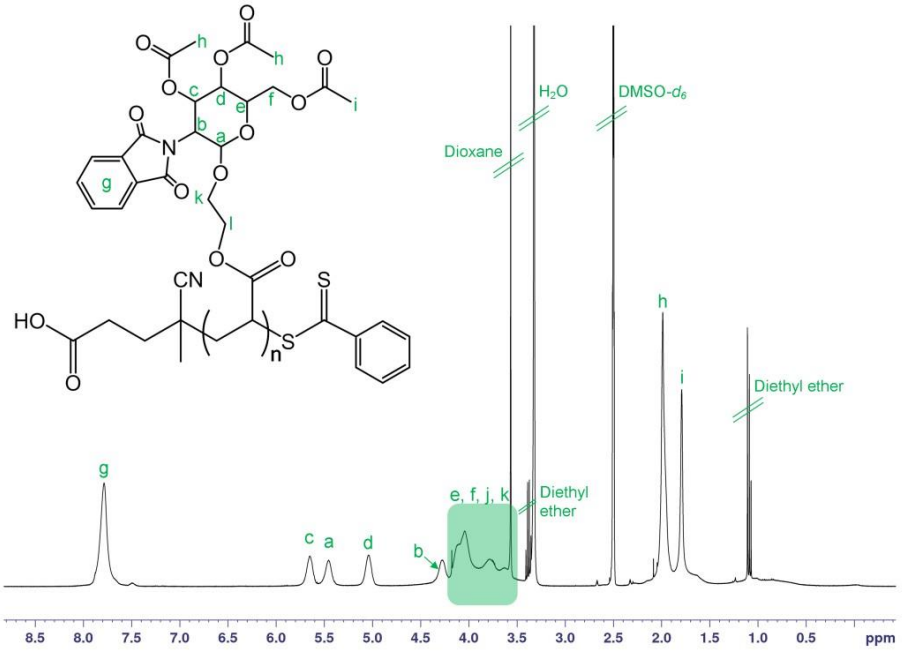

Figure S15: ^{1}H-NMR spectra of P(GluPAHEA) homopolymer obtained in DMSO-d_6 and at 400 MHz

D-glucopyranoside (GluCF$_3$HEA) in $CDCl_3$ and at 400 MHz.

Kinetics Study of the polymerisation of GluCF$_3$HEA

GluCF$_3$HEA (700 mg, 1.40 × 10^{-3} mol) and CPADB (7.8 mg, 2.79 × 10^{-5} mol) were added to a flask and dissolved in 600 μL of DMAc. ACCN was then added to the solution (137 μL of 10 mg/mL solution, 5.61 × 10^{-6} mol) and mixed thoroughly. The final volume of the reaction solution was 1.17 mL, with a monomer concentration of 1.20M. The molar ratio of GluCF$_3$HEA : CPADB : ACCN was designed to be 50 : 1 : 0.2. The flask was sealed and degassed with N_2 for one hour before reaction was initiated by heating the sample to 85 °C. Samples were removed at regular intervals over an 11 h period and

analysed via ^{1}H-NMR and GPC to determine the conversion and polydispersity, respectively. The stacked GPC traces of the polymers taken at various timepoints are shown in Figure S16 and Figure S17 shows the concentration of radicals throughout the study. Characteristics of each of the polymers obtained throughout the kinetics study are listed in Table S2.

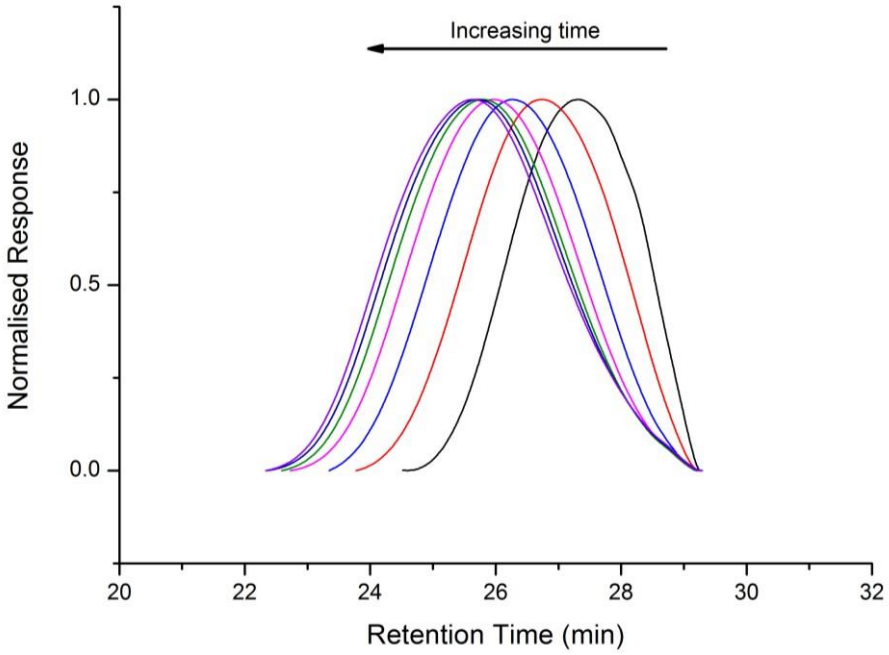

Figure S16: Stacked GPC traces of P(GluCF$_3$HEA) at various conversions after polymerisation at 85 °C using CPADB as a RAFT agent

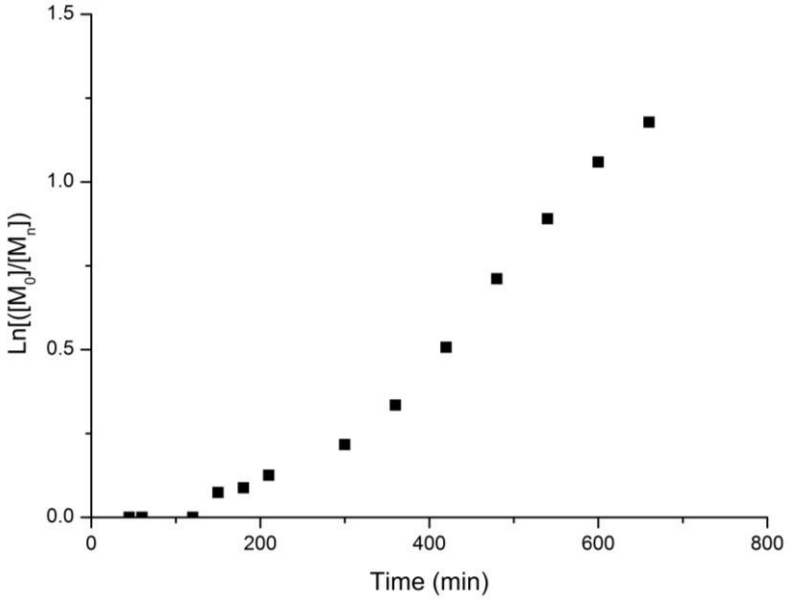

Figure S17: Concentration of radicals with time in the polymerisation of P(GluCF$_3$HEA) at 85 °C and with CPADB as the RAFT agent. Monomer concentration calculated from ^{1}H-NMR of crude reaction mixtures.

Table S2: Characteristics of P($GluCF_3HEA$) obtained at various timepoints in the kinetics study of $GluCF_3HEA$ polymerisation

Time Point (min)	Conversion (%)[a]	Theo. Mn[a] (g/mol)	Mn[b] (g/mol)	Đ[b]
300	19	5 100	5 600	1.24
360	28	7 400	7 100	1.27
420	40	10 200	8 700	1.27
480	51	13 000	9 900	1.28
540	59	15 000	10 500	1.29
600	65	16 600	10 800	1.31
660	69	17 600	11 000	1.32

[a]Calculated by NMR, [b]calculated by GPC

Homopolymerisation of $GluCF_3HEA$ for chain extension

A general procedure for the polymerisation of $GluCF_3HEA$ is outlined as follows. $GluCF_3HEA$ (300 mg, 6.01×10^{-4} mol), CPADB (3.4 mg, 1.20×10^{-5} mol) and ACCN (58.7 μL of 10 mg/mL solution, 2.40×10^{-6} mol) were added to a flask and dissolved in DMAc (250 μL). The molar ratio of $GluCF_3HEA$: CPADB : ACCN was designed to be 50 : 1 : 0.2. The contents were mixed gently until all of the monomer dissolved. The final volume of the solution was *c.a.* 480 μL, which gave a monomer concentration of 1.25 M. The flask was sealed and degassed for 30 min with N_2 before it the polymerisation was initiated by placing the flask in an oilbath at 85 °C. The polymerisation was allowed for proceed for 6 h and the reaction subsequently quenched by introducing air into the system. The polymer was isolated via precipitation in diethyl ether (2 ×) and the solvent then removed under reduced pressure at 40 °C. Conversion was determined via ^{1}H-NMR and the polydispersity of the polymer was determined via GPC analysis. Final polymer composition was determined to be $P(GluCF_3HEA)_{30}$.

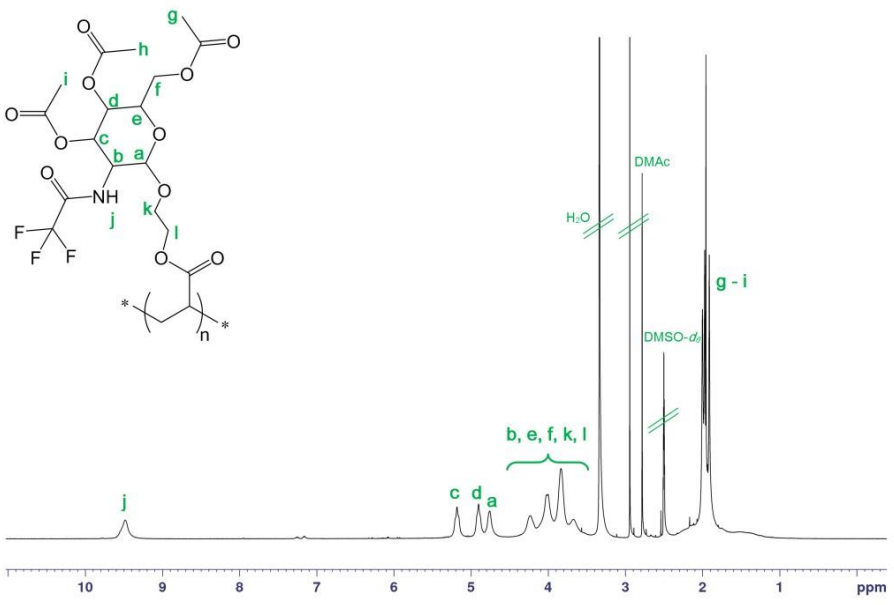

Figure S18: ^{1}H-NMR spectra and assignment of P(GluCF$_3$HEA) in DMSO-d_6 and at 400 MHz.

Table S3: Characterisation of P(GluCF$_3$HEA)$_{30}$ homopolymer

Polymer	Conversion[a] (%)	Theo. Mn[a] (g/mol)	Mn[b] (g/mol)	Đ[b]
P(GluCF$_3$HEA)$_{30}$	60	15 300	9 400	1.31

[a]Calculated by NMR, [b]calculated by GPC

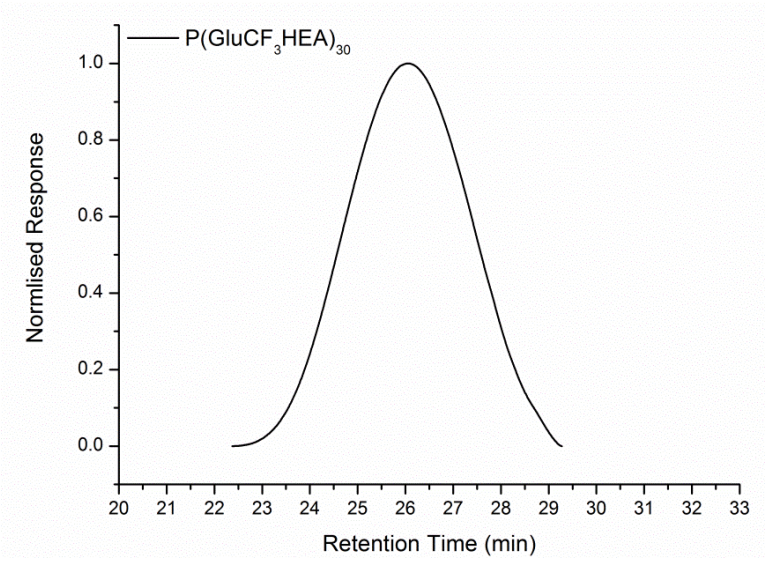

Figure S19: GPC trace of P(GluCF$_3$HEA)$_{30}$ homopolymer

Homopolymerisaton of butyl acrylate for macroRAFT synthesis

Poly(butyl acylate) (PBA) was synthesised via the bulk polymerisation of butyl acrylate. Butyl acrylate (1.50 g; 1.17×10^{-2} mol) was de-inhibited by passing through a column of basic alumina immediately before use. BSPA (15.9 mg; 5.84×10^{-5} mol) and AIBN (1.0 mg; 6.2×10^{-6} mol) were added to the monomer and the mixture vortexed until all reagents had dissolved. Oxygen was removed from the polymerisation solution via three freeze-pump-thaw cycles before the vessel was filled with nitrogen and sealed. The polymerisation was allowed to proceed at 60 °C for 90 min before the reaction quenched by simultaneously cooling the vessel and exposing the solution to atmosphere. The polymer was isolated via precipitation in methanol and dried under reduced pressure at 40 °C for 24 hr. Conversion was determined via ^{1}H-NMR and polydispersity was determined via GPC analysis.

Chain extension of PBA_{119} macroRAFT with $GluCF_3HEA$

PBA_{119} (197.6 mg; 1.27×10^{-5} mol), $GluCF_3HEA$ (299.7 mg; 5.22×10^{-4} mol) and AIBN (14.1 μL of 10 mg/mL solution; 8.71×10^{-4} mol) was added to DMF (750μL). Although the macroRAFT was initially soluble, once all the monomer had dissolved, the solution also underwent phase separation as PBA_{119} was no longer soluble. Further DMF (200 μL) was added to the mixture and the vessel sealed and degassed for 60 min. The reaction was left at 60 °C for 24 hr before the solvent was removed via dialysis against water. The crude polymer was then isolated via lyophilisation. Conversion was determined via ^{1}H-NMR and polydispersity was determined via GPC analysis.

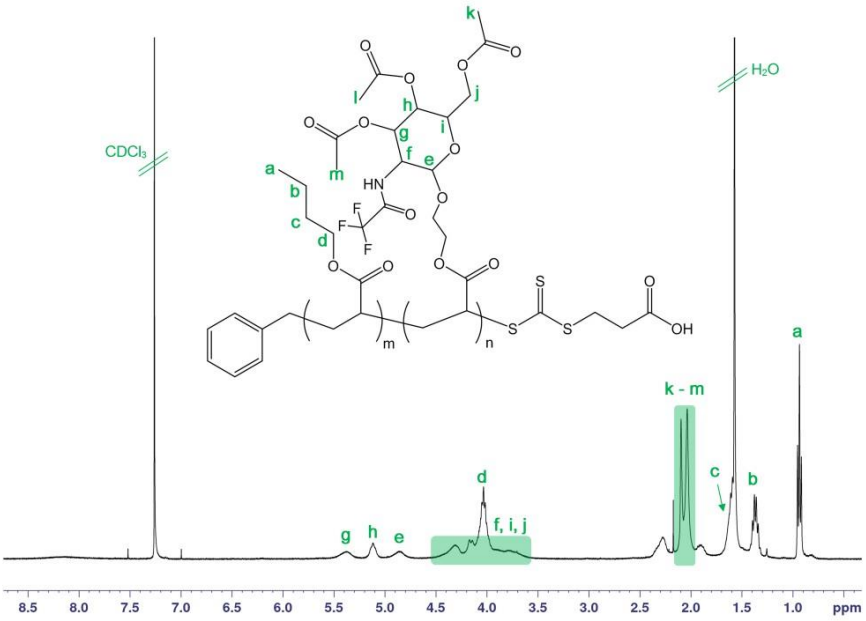

Figure S20: ^{1}H-NMR spectra of PBA-b-P($GluCF_3HEA$) block copolymer in $CDCl_3$ and at 400MHz

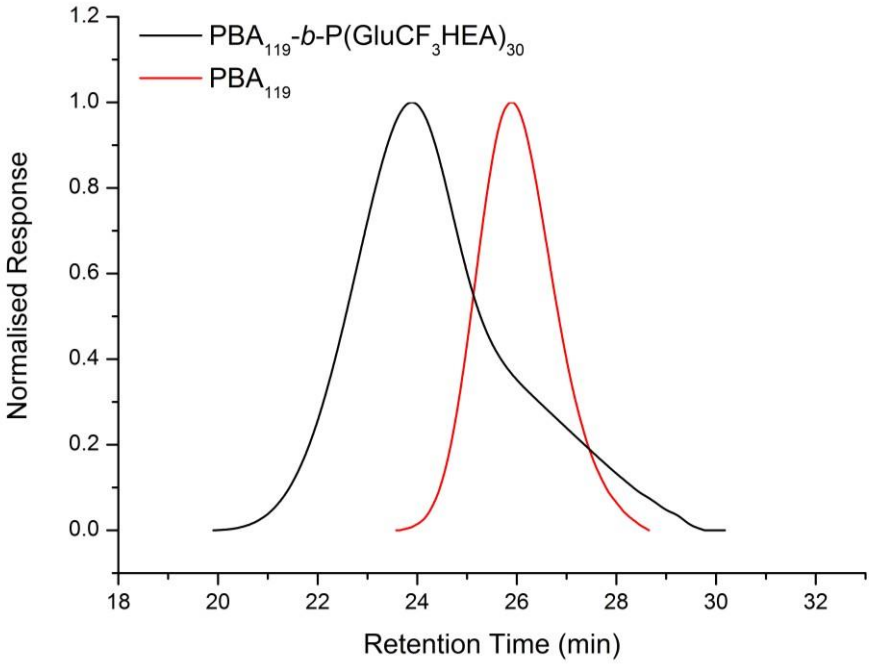

Figure S21: GPC trace of macroRAFT agent PBA_{119} (red) and the chain extended block copolymer PBA_{119}-*b*-$P(GluCF_3HEA)_{30}$ (black)

Deprotection[6] of $P(GluCF_3HEA)_{30}$ homopolymer

$P(GluCF_3HEA)_{30}$ (49.9 mg, 6.63×10^{-6} mol) was dissolved in chloroform : methanol (10 : 1, v/v, 20mL) and the solution purged with nitrogen for 10 min. Sodium methoxide (25 wt%) was diluted in anhydrous methanol (1 : 10 dilution) immediately before dropwise addition (230 μL of diluted sodium methoxide solution) to the polymer solution. The ratio of protecting groups on each sugar unit (inclusive of the trifluoroacetamide protecting group) to sodium methoxide was 4 : 1. The solution was stirred at RT for 3 h before the solvent was removed under reduced pressure. The polymer was further purified via dialysis against milliQ water (MWCO = 3.5 kDa) and isolated via lyophilisation to give a white powder.

The polymer was redissolved in MilliQ water (1mL) and $NaHCO_3$ (1mL of 50 mg/mL solution in MilliQ water) and the solution stirred at RT for 24 h. After 24 h, the polymer was purified via dialysis against MilliQ water (MWCO = 3.5 kDa) and isolated via lyophilisation to give a white powder. As ^{19}F-NMR indicated that deprotection of the amine was not complete, the polymer was redissolved in DMSO (1 mL) and $NaHCO_3$ (50.0 mg in 3 mL of MilliQ water) added to the solution. The solution was stirred at RT for 24 h before the polymer (PGluHEA) was again purified via dialysis against milliQ water (MWCO = 3.5 kDa) and isolated via lyophilisation. ^{19}F-NMR indicates that deprotection was 70% successful (relative to internal standard of pentafluoro styrene acrylate) and the homopolymer was used as is for cytotoxicity testing. The homopolymer will be referred to as P(GluHEA).

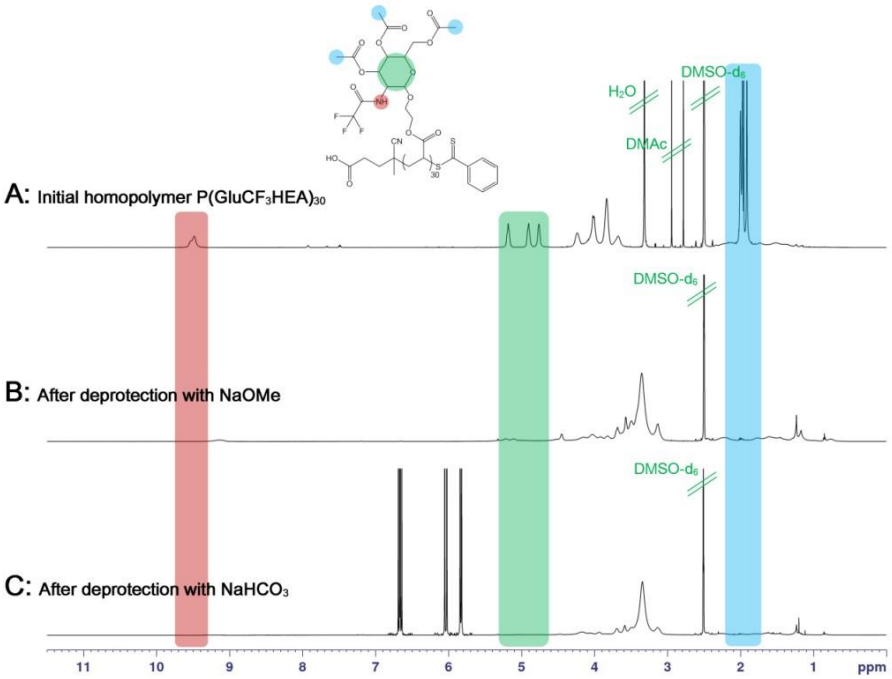

Figure S22: Stacked ^{1}H-NMR spectra of P(GluCF$_3$HEA)$_{30}$ before deprotection reactions (spectra A), after first deprotection reaction using NaOMe (spectra B) and after second deprotection reaction with $NaHCO_3$ (spectra C).

Deprotection of PBA$_{119}$-*b*-P(GluCF$_3$HEA)$_{30}$

PBA$_{119}$-*b*-P(GluCF$_3$HEA)$_{30}$ (102.2 mg; 3.39 × 10^{-5} mol) were dissolved a mixture of chloroform and methanol (10 : 1, v/v) and the solution degassed with nitrogen for 30 min. Sodium methoxide (25wt% in methanol, 290 μL) was diluted in anhydrous methanol to give a solution with a final volume of 1 mL and this solution was prepared immediately before use. Under nitrogen, the diluted sodium methoxide solution (100 μL; 1.24 × 10^{-4} mol) was added dropwise the polymer solution with stirring. The solution was then left to stir at RT for 3 hr, whereby the solution had turned slightly opaque. Excess sodium methoxide and solvent was removed via dialysis against MilliQ water to give a clear, colourless solution.

The clear solution was transferred to a roundbottom flask where $NaHCO_3$ (100 mg; 1.19 × 10^{-3} mol) was added. The vessel was sealed and agitated in an orbital shaker for 48 hr at 35 °C whereby the solution had turned slightly opaque again. Excess $NaHCO_3$ was removed via dialysis against MilliQ water and the block copolymer isolated via lyophilisation. Success of the acetyl and trifluoroacetoamido deprotection steps was confirmed using ^{1}H-NMR and ^{19}F-NMR, respectively.

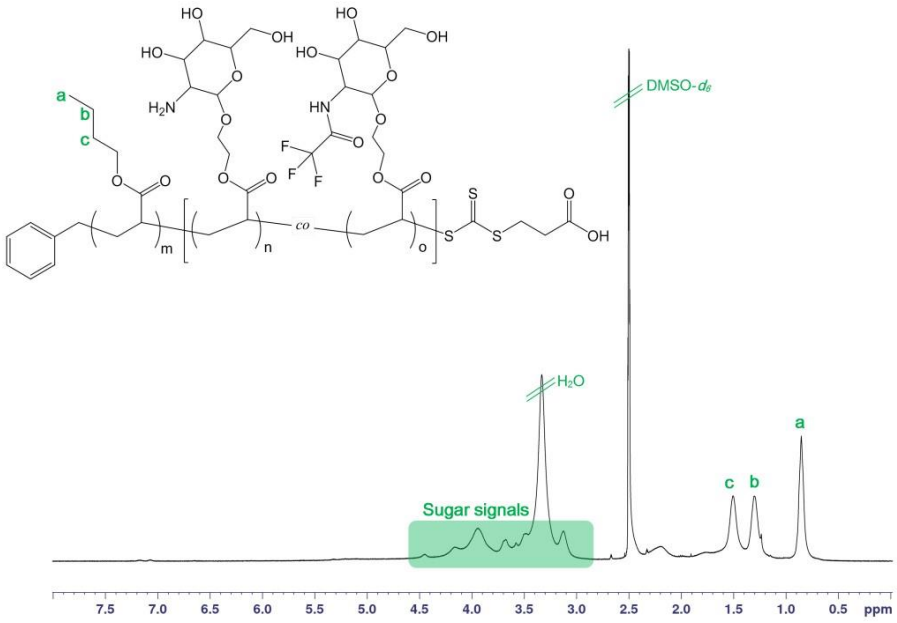

Figure S23: ^{1}H-NMR spectrum of block copolymer PBA-b-P(GluHEA) after deprotection with both NaOMe and $NaHCO_3$. Spectrum obtained in DMSO-d_6 and at 400 MHz

Micellisation of PBA_{119}-*b*-P[$(GluHEA)_{26}$-*co*-$P(GluCF_3HEA)_4$]

From the crude solution of fluorescent polymer in DMSO after FITC attachment, micellisation of the particles were prepared without purification of the polymer from unreacted FITC. To a sealed vial containing the crude reaction solution, MilliQ water was added slowly with vigorous stirring (11 mL; 0.85 ml/hr) under darkness. After water addition, the clear and fluorescent green solution was transferred to a dialysis bag (MWCO: 3.5 kDa) and dialysed against MilliQ water (adjusted to *ca*. pH 8 using NaOH). The water was changed until the waste water did not have a fluorescence reading. The particles (M-30-F) were dialysed once against MilliQ water at *ca*. pH 6 (adjusted using HCl) before being dialysed twice against MilliQ water (pure) to finish purification process. All dialysis steps were performed under darkness.

A nonfluorescent micellar (M-30) sample was also prepared for cytotoxicity studies, DLS measurements and zeta potential measurements. PBA_{119}-*b*-P[$(GluHEA)_{26}$-*co*-$P(GluCF_3HEA)_4$] (10.4 mg) was dissolved in DMSO (1 mL) in a flask fitted with a stirrer bar to give a pale brown solution. MilliQ water was slowly added to the solution with vigorous stirring (4 mL; 0.25mL/hr). Once all the water had been added, the clear and slightly brown solution was transferred to a dialysis bag (MWCO: 3.5 kDa) and purified of the solvent by dialysing against MilliQ water.

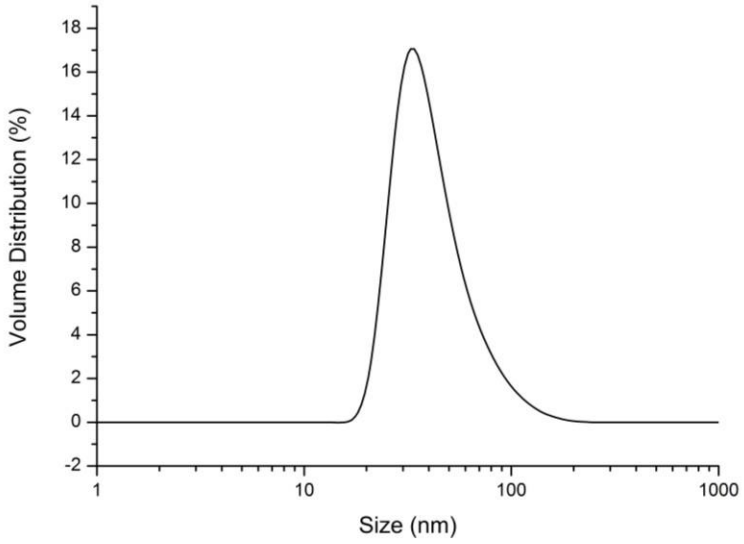

Figure S24: DLS trace of the volume distribution of M-30 micelles measured in water and at 25 °C

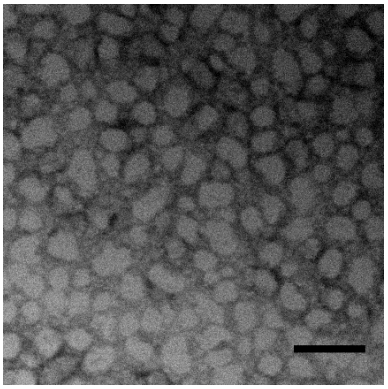

Figure S25: TEM image of M-30-F micelles after staining with UA for 2 min. Scalebar = 100 nm

FITC attachment to P(GluHEA), chitosan, and PBA_{119}-b-P[$(GluHEA)_{36}$-*co*-P($GluCF_3HEA)_4$]

In brief, for P(GluHEA) and chitosan, a stock solution of FITC in DMSO (1 mg/mL) was prepared immediately before use. P(GluHEA) (3.0 mg) was dissolved in DMSO (1mL) before FITC (30uL of stock solution) was added and the solution stirred under darkness for 24 hr. Similarly, chitosan (3.6mg) was dissolved in 1% acetic acid (400 μL) before the solution was diluted with DMSO (1mL). FITC (36 μL) was added) and the solution stirred under darkness for 24 hr. For PBA_{119}-*b*-P[$(GluHEA)_{26}$-*co*-P($GluCF_3HEA)_4$, the concentration of the FITC stock solution was 0.06 mg/mL. the copolymer was dissolved in DMSO (3 mL) before FITC (250 μL of stock solution) was added and the solution stirred under darkness for 24 hr. P(GluHEA) and chitosan were purified via dialysis against MilliQ water, adjusted to *ca*. pH 8 using NaOH (MWCO: 3.5 kDa). The last solvent change was against pure MilliQ water to remove excess NaOH and the two samples were isolated via lyophilisation. PBA_{119}-*b*-

P[(GluHEA)$_{26}$-*co*-P(GluCF$_3$HEA)$_4$ and FITC crude reaction solution in DMSO was used without modification for micellisation.

Caco-2 cell culture

Human Caco-2 cells (colon adenocarcinoma) (Sigma Aldrich Australia) were cultured in DMEM, supplemented with 10% fetal bovine serum (FBS), 1 mM non-essential amino acids, 2 mM GlutaMAX™ and 2.5 μg/mL Plasmocin as an antibiotic agent. The cells were cultured at 37 °C and 5% CO_2. Cells were passaged once they reached 70 – 80 % confluence by trypsin/EDTA treatment (3 – 5 min) to collect the cells and subsequent seed in a new tissue culture flask. All studies were conducted on cells which have been passaged less than 30 times.

Cytotoxicity testing against Caco-2 cells

Caco-2 cells were seeded at a density of 7 × 10^3 cells/well in 96 well tissue culture plates and incubated at 37°C and 5% CO_2 for 24 hr. The media from the plates were then discarded and 100 μL of 2x DMEM added to each well. 100 μL of sterile MilliQ water were added to the control wells, whilst 100 μL of the sample solution were added to each relevant well to give a total volume of 200 μL in all wells.

P(GluHEA) was dissolved in MilliQ water (2 mg/mL) and the solution was sterilised via filtration though 0.45 μm membrane. The initial P(GluHEA) solution was serially diluted, with the highest concentration tested being 1 mg/mL. A stock solution of M-30 in MilliQ water (2 mg/mL) was prepared in a similar manner and also sterilised via filtration (0.45 μm).

After 72 hr incubation with the two polymeric samples, Caco-2 cells were crosslinked by treatment with cold trichloroacetic acid (TCA, 10%) for 30 min at 4°C. The cells were washed with water (5 ×) and stained with sulforhoadmine B (SRB) solution (0.4 wt% in 1% acetic acid) for 20 min under darkness at RT. The dye was discarded and plates washed with 1% acetic acid (5 ×) before air drying. Tris(hydroxymethyl)aminomethane (TRIS) buffer was added to each well (200 μL) and left for 5 min to dissolve the bound SRB. The absorbance at 490 nm was measured. The cytotoxicity of each polymer sample was conducted in quadruplicate, where the IC_{50} data is represented as mean ± standard deviation, where n = 4.

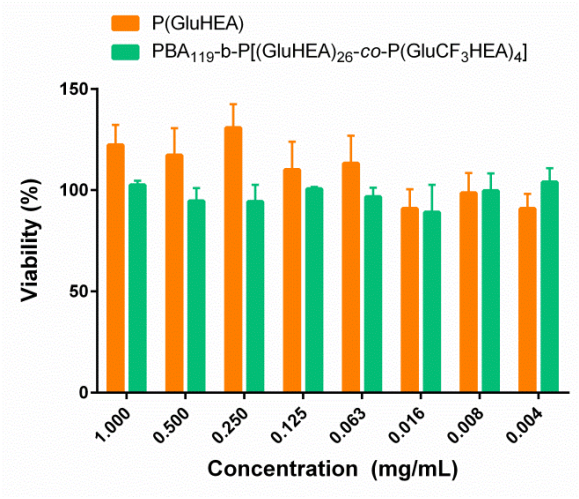

Figure S26: Cytotoxicity study results of P(GluHEA) and M-30 against Caco-2 after 72 hr incubation

Formation and visualisation of Caco-2 monolayer

Caco-2 cells were seeded at a density of 10 000 cells/cm^2 in a 35mm Fluoro dish and cultured for 14 days. The media were discarded and cells washed three times with PBS. The cells were then crosslinked with 4% paraformaldehyde for 20 min at 4 °C. The cells were washed three times with PBS –T (PBS containing 0.1% Tween-20), with each wash taking 3 min. The cells were blocked using 10% donkey serum (in PBS-T) for 1 hr at 37 °C and then rinsed with PBS-T. The cells were incubated overnight and at 4 °C with the first antibody, an anti-ZO1 tight junction protein antibody (rabbit polyclonal, diluted 1 : 50 in 1% BSA in PBS-T).

After rinsing with PBS-T, the cells were incubated with the second antibody, Alexa Fluor® 568 conjugated Donkey anti-Rabbit IgG (H+L) secondary Antibody (1 : 200 dilution in PBS containing 1% BSA and 0.1% Tween-20), for 2 hr at 37 °C. After incubation, the cells were washed three times with PBS-T, with each wash taking 3 min. The cells were then imaged under the confocal microscope.

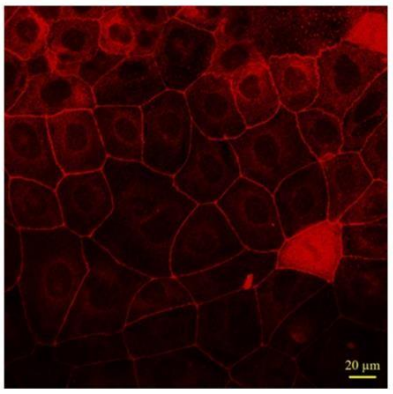

Figure S27: Confocal image of Caco-2 monolayer after 14 days culture. Tight junction ZO-1 protein (red) indicates successful monolayer formation. Scalebar = 20 µm

Deacetylation of Chitosan[7]

LMW Chitosan (5027.4 mg, Sigma Aldrich) was suspended in NaOH (100 mL, 50 w/v%) and fitted with a condenser. The mixture was heated at 85 °C for 7 h before the mixture was cooled and diluted in 500 mL of cold MilliQ water. The off-white solids were collected by centrifugation, the brown aqueous layer discarded and the solids resuspended in methanol : water (4 : 1, v/v). After vigorous shaking, the solid was again collected by filtration and the liquid layer discarded. The washing cycle was repeated until the liquid layer was neutral. Residue solvent was removed at 40 °C under vacuum. The dried deacetylated chitosan was dissolved in 1 % (v/v) acetic acid and dialysed against the same solution (MWCO: >25 kDa, 3 × solvent change) before dialysis against MilliQ water (1 × solvent change) to remove excess acetic acid. The fraction was isolated via lyophilisation. Degree of deacetylation was confirmed via ^{1}H-NMR. Initial degree of deaceylation of commercial chitosan (LMW) was calculated by comparing the integral of peak g* to sum of integral of a and a* (Figure S28). Degree of deacetylation was calculated to be *ca*. 19 %. The same ratio was recalculated after deprotection and compared to initial percentage to give degree of deacetylation of the final product to be *ca.* 2 %.

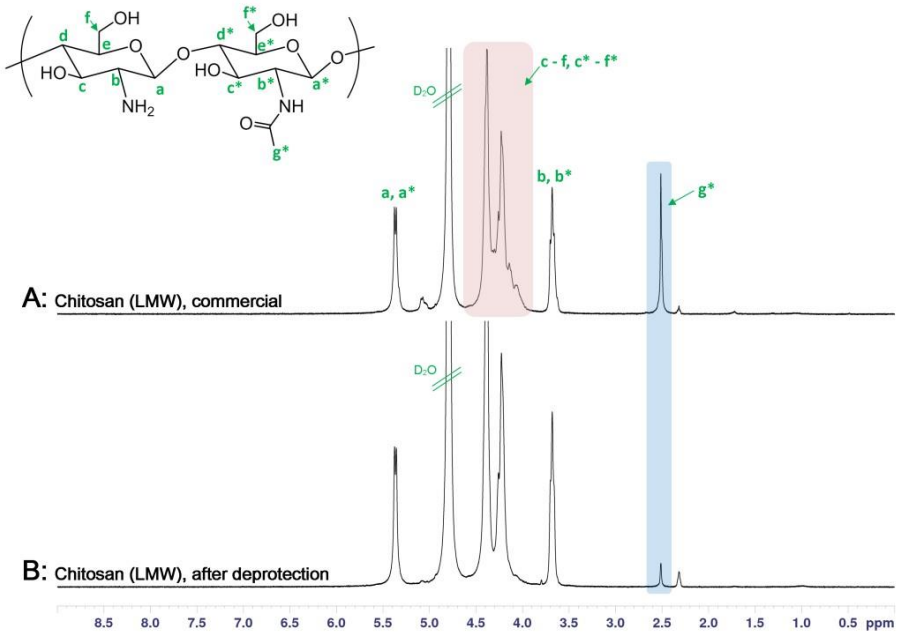

Figure S28: Stacked ^{1}H-NMR spectra of commercial sample of chitosan (LMW) before deprotection (spectra A) and after deprotection using NaOH and purification (spectra B). All spectra obtained in D_2O (containing 2% DCl), at 70°C and at 400 MHz

Migration studies of Lucifer Yellow, chitosan, P(GluHEA) and PBA_{119}-*b*-P[$(GluHEA)_{26}$-*co*-P($GluCF_3HEA)_4$] through Caco-2 monolayer

Caco-2 cells were seeded in Corning Transwell membrane cell inserts (Pore size = 0.4 μm) for 24 well plates at a density of 10 000 cells/cm^2. The cells were cultured at 37 °C and 5% CO_2 for 21 days before use, where the growth media was changed every 48 hr. All samples to be tested were sterilised via filtration (0.45 μm). Samples were prepared in media with final concentration of 50 μg/mL. 200 μL of each sample solution was carefully loaded on the apical side in each insert. HBSS buffer (600 μL), supplemented with HEPES[8] (25 mM), was loaded into each well beneath the inserts on the basolateral side. The inserts were then incubated under darkness for 48 hr. After the incubation, the solution from both the apical side and basolacteral side were removed and the fluorescence measured.

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