Supporting Information: Mapping Protein-Polymer Conformations in Bioconjugates with Atomic Precision

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Materials and Methods

General Procedures

Determination of Polymer Molecular Weight Parameters via Size Exclusion Chromatography. Size exclusion chromatography (SEC) was performed to determine polymer molar mass data using an Agilent 1260 SEC system equipped with an auto-sampler, an Agilent 1260 isocratic pump, Agilent 1 guard and 2 analytical PolarGel-M columns, degasser and Agilent 1260 refractive index (RI detector). DMF with 0.1% LiBr was used as the eluent at 50 °C with a flow rate of 1 mL/min. All the samples were filtered with a 0.22um syringe filter before the injection to instrument. The system was calibrated with poly(methyl methacrylate) standards with molecular weight ranges of the range of 617500 to 1010.

Pierce copper assay for protein concentration. Protein concentrations were obtained using the standard protocol for the Thermo-Fisher Pierce reagent in a 96-well plate. Briefly, 150uL of Pierce reagent (Thermo product #22660) was mixed with 10uL of protein sample, and analyzed on a Biotek Synergy H1 microplate reader. The plate was calibrated with pre-diluted BSA standards (Thermo product #23208) ranging from 0.125g/L to 2.0g/L.

One-pot synthesis of 2-(ethylthiocarbonothioylthio)-propionic acid (PAETC). PAETC was synthesized in one pot according to our previously reported procedure.[1] Potassium hydroxide (14.6g, 260mmol) was dissolved in 15mL distilled water. This solution was added dropwise to a solution of ethanethiol (18.6ml, 258mmol) in acetone (150ml) in a round bottom flask, while stirring on ice. Then, carbon disulfide (17.1ml, 284mmol) was added, and the solution was stirred on ice for 30 minutes. The flask was then removed from ice and 2-bromopropionic acid (23ml, 256mmol) was added dropwise. Then, the solution was stirred at room temperature for 18h. The acetone in the flask was then removed under reduced pressure in a rotary evaporator. The oily product which remains was then dissolved in 200ml ethyl ether, and transferred to a separatory funnel. The ether layer was washed with water (200ml) 10 times, followed by one wash with brine (200ml). Then, the ether was removed on a rotary evaporator, resulting in a red oil, which was transferred to tared 20ml glass vials and stored at -20°C, where the product became an opaque yellow solid overnight (42.2g, 201mmol, 79% yield). The product purity was confirmed by NMR. (300MHz, CDCl3, δ ppm): 4.87 (1H, q, J = 7.4 Hz, $CH_3CH(S)$-COOH), 3.38

(2H, q, J = 7.4 Hz, CH_3CH_2S), 1.63 (3H, d, J = 7.4 Hz, $CH_3CH(S)COOH$), 1.36 (3H, t, J = 7.4 Hz, CH_3CH_2S).

Synthesis of N-(2,2,6,6-tetramethyl-4-piperidinyl)-2-propenamide (TMPA-HCl). The procedure used is a simplified adaptation of a previous report.[2] In a 200mL round bottom flask, 4-amino-2,2,6,6-tetramethylpiperidine (10g, 10.965mL, 64.0mmol) was mixed with 81.5mL of toluene dried over molecular sieves. The flask was sealed with a rubber septum, placed on ice, and deoxygenated by bubbling with nitrogen gas for 15m. Then, acryloyl chloride (5.33mL, 65.9mmol) was added dropwise by syringe, with rapid stirring. The flask was stirred for 1h on ice, followed by 1h at room temperature. The crude product, a white precipitate, was filtered and dried on a Hirsch funnel. Once dry, the product was recrystallized by dissolving in minimal hot water (around 30ml), followed by slow cooling to room temperature and then storing at 4°C for several days. The solids were dried on a Hirsch funnel, yielding granular, colorless crystals (7.5g, 36mmol, 67% yield). The product purity was confirmed by NMR. (300MHz, D_2O, δ ppm): 6.12 (2H, m, J = 4.3Hz, CH_2CH-CO), 5.67 (1H, dd, J_1 = 7.7Hz, J_2 = 4.0Hz CH_2CH-CO), 4.26 (1H, tt, J_1 = 12.3Hz, J_2 = 3.6Hz $NHCH(CH_2)$-CH_2), 1.98 (2H, dd, J_1 = 13.8Hz, J_2 = 3.6Hz $NHCH(CH_2)$-CH_2), 1.45 (2H, dd, $J_{1,2}$ = 13.0Hz $NHCH(CH_2)$-CH_2) 1.43 (6H, s), 1.33 (6H, s).

Polymer and small-molecule nuclear magnetic resonance spectroscopy. Spectra for PAETC, TMPA, and all polymers were collected on Bruker DPX or Avance 200, 300, or 500 MHz NMR instruments using appropriate deuterated solvents (Cambridge Isotope Laboratories). Number average molecular weight and chain-length data were collected only on the 500 MHz spectrometer. Chain-lengths were estimated by integrating the resonances on the PAETC end groups (2 proton quartet at ~3.3 ppm $CH_3\underline{CH_2}SCSS$), against the DMAm (6 proton peaks at 3.0-2.7 ppm from $N(\underline{CH_3})_2$) and TMPA (1 proton ~3.8 ppm from $\underline{CH}NHCO$) repeat units in the polymer to determine the mean number of DMAm units (u_{DMAm})and TMPA units (u_{TMPA}) per block polymer as estimated by NMR. This was performed after each chain extension to evaluate the composition of each block. *M*n by NMR was estimated by Equation S1:

$$M_{n-NMR} = M_{PAETC} + M_{DMAm} \times u_{DMAm} + M_{TMPA} \times u_{TMPA} \quad \text{(S1)}$$

Where M_{CTA}, M_{DMAm}, M_{TMPA} are the molecular weights of PAETC, DMAm and TMPA, respectively.

Protein and conjugate nuclear magnetic resonance spectroscopy. All protein NMR spectra were collected at 25°C (298K) on a Bruker 600 MHz Avance III spectrometer equipped with a 5 mm triple resonance (TXI) probe. ^1H-^{15}N HSQC spectra were acquired using the Bruker hsqcetf3gpsi pulse program using 2048 (proton) and 512 (nitrogen) complex points. All spectra of bioconjugates were acquired with 16 scans. Spectra were processed using NMRPipe[3] and visualized using Sparky[4]. Initial chemical shift assignments were pulled from the BMRB (entry 15410)[5] and adjusted for pH and conjugation effects.

Polymer Preparation and Conjugation

Example block copolymer synthesis (B-block). To a 50ml round-bottom flask was added DMAm (2.5g, 25.2mmol), PAETC (354mg, 1.68mmol), VA-044 (10.9mg, 33.7umol), and 2.46g of a 1:1 water:methanol solution. The flask was sealed with a rubber septum, and deoxygenated for 20 minutes using nitrogen gas. Then, the flask was lowered into a 65°C oil bath for 3 hours. Two small aliquots of this first block were taken – one to check the monomer conversion by NMR, the other to be dried *in vacuo* for SEC and higher resolution NMR later.

With over 95% conversion, the next block was added as follows. TMPA-HCl (1.66g, 6.72mmol) was weighed out and added to the flask containing the polymer, followed by the minimal volume of 1:1 water:methanol mixture required to dissolve the solid TMPA-HCl (around 10ml). Dissolution was assisted by stirring and sonication. Once the monomer had dissolved, VA-044 initiator (10.9mg, 33.7umol) was added. Then, the flask was sealed and deoxygenated for 20 minutes, followed by 18h at 65^{o}C. Once again, 2 aliquots were taken for NMR and SEC. With over 95% conversion, the final block was added as follows. DMAm (2.5g, 25.2mmol), and VA-044 (10.9mg, 33.7umol) were added and the flask was sealed and deoxygenated for 20m. Then the flask was heated to 65^{o}C for 3h. 2 aliquots were again taken. With sufficient conversion, the polymer was then dried on a rotary evaporator for 1h, then moved to a vacuum oven and dried under reduced pressure with mild (ca. 50^{o}C) heat. High methanol content in the polymer can reduce the efficiency of the oxidation step; to confirm uniform elimination of methanol, the dried polymer was pulverized using a mortar and pestle, yielding a fine yellow powder. Then, NMR was performed to assess acceptable methanol content – generally, less than 0.1 equivalents versus PAETC.

Example oxidation of block copolymer with mCPBA (B-block). To four, numbered, glass 20ml vials was added dry polymer powder (125mg, 30.0umol). The polymer was dissolved with stirring and mild (<50^{o}C) heat using 1.12ml tert-butanol. Once dissolved, the vials were protected from ambient light by wrapping with duct tape or electrical tape. One vial at a time, mCPBA (58.8mg, 263umol when accounting for added stabilizers) was weighed out and added, followed immediately by 2M NaOH (240uL, 480umol). Then, the vials were stirred at 200rpm for 1h at room temperature, using 1/2” by 5/6” Teflon-coated magnetic stir bars. The oxidation results in viscous scarlet solutions. The yield of oxidation was measured by EPR on a Bruker NanoEMX benchtop EPR instrument, calibrated using TEMPO standards up to 300uM. The vial containing the polymer with highest oxidation yield (typically 55-60%) was then quantitatively transferred to a 0.5-3ml capacity, 2kDa MWCO dialysis cassette. A small amount (<1ml) of t-butanol was used to rinse the vial, and this was also added to the cassette. Then, the polymer was dialyzed at 4^{o}C into 3L of 10mM sodium dodecyl sulfate (SDS), followed by another 3L of 10mM SDS, followed by 3L of water, followed by another 3L of water, exchanging approximately every 12 hours. Upon completion, the polymer was transferred to a 3kDa MWCO centrifugal filter. A 100uL aliquot was taken and combined with 400uL D_2O in an NMR tube to confirm removal of mCPBA, meta-chlorobenzoic acid, and SDS. Then, the polymer solution was concentrated in the centrifuge at 4^{o}C to ca. 600uL, which required up to 17,400RCF, and EPR was performed on the colorless filtrate to confirm no significant loss of polymer. The concentration of polymer in the upper solution was calculated by measuring its spin label concentration by EPR and then factoring in the oxidation yield and average number of TMPA per chain. After the concentration was determined to be high enough, the polymer solution was transferred to a dark brown Eppendorf tube and stored at -80^{o}C until use. The spin labels on polymers prepared and stored in this way show no decay for at least 40 days.

Example polymer conjugation to ubiquitin (B-block). ^{15}N-ubiquitin, purchased from LifeSensors (catalog# NS101), was prepared by dissolving the lyophilized powder in 10mM phosphate buffer (PB) at pH 7.5. Directly before use, the protein concentration was obtained by Pierce copper assay. The polymer was prepared by thawing and centrifuging at 17,000G for 20 minutes. To an autoclaved 1.5mL Eppendorf tube covered with tape were added 0.1M PB at pH 7.5 (80.6uL), N-hydroxysuccinimide (NHS) (4.57uL of a 40g/L PB solution – 1.6umol), and oxidized B-block solution (223uL, 9.33umol). Then, EDC-HCl (7.16mg, 37.3umol) was added,

followed by several seconds of vortexing. The tube was then quickly centrifuged to bring down liquid from the sides. Then, immediately, ^{15}N-ubiquitin (192.3uL, 233umol) was added and the tube was rotated at room temperature for 2 hours. Other conjugates differ only in the amount of EDC-HCl used; B-block is 160eq vs ubiquitin, while A-block and C-block required 80eq, 0-block required 120eq, to reach sufficient conjugation, as determined by SDS-PAGE.

NMR sample preparation. Conjugate (360uL) was transferred to an autoclaved 1.5ml Eppendorf tube, followed by D_2O (40uL), and mixed. All the sample was transferred to a D_2O susceptibility-matched BMS-005B Shigemi NMR tube, and sealed with the corresponding matched plunger and parafilm.

Synthesis and Conjugation of DP5 DMAm. A short DMAm polymer was synthesized in the same way as the first step of A-block, targeting 5 DMAm units. Rather than purifying by trituration, which biases the products towards higher chain lengths, the polymer was dried under reduced pressure for several days. The polymer was then used for conjugation. To a 1.5ml Eppendorf tube was added 5.2mg (7.4umol) DP5 DMAm, which was dissolved in 100mM phosphate buffer (pH 7.5, 126.2uL), and NHS (3.62uL, 1.3umol). Then, EDC-HCl (1.4mg, 7.4umol) was weighed out and immediately added. The tube was vortexed and centrifuged briefly to dissolve the EDC, and then ^{15}N Ub (92.5nmol) was added and the tube was rotated at room temperature for 2h. Once complete, the conjugation solution was added to 3kDa MWCO dialysis tubing and dialyzed with ultrapure water over 24h and room temperature. Then, the conjugate was concentrated to 200uL using a centrifugal filter, and stored at 4^{o}C until used for trypsin digestion.

Chemical Denaturation by Circular Dichroism. Using conditions adapted from Ibarra-Molero et al.,[6] we compared the stability of native ubiquitin and ubiquitin conjugated with different densities of C-block. Samples were prepared by mixing sodium acetate buffer (10mM, pH 4), and guanidine-HCl (8M in the same acetate buffer) in autoclaved 1.5ml Eppendorf tubes to the desired molarity (0.4M increments from 0M up to 7.2M when necessary). Immediately before running each sample on the CD instrument, ubiquitin or conjugate was added, bringing the final volume up to 340ml, and a protein concentration of 6.59uM. Before each run, the CD cuvette (1mm pathlength) was rinsed three times with distilled water and patted dry with a lint-free chemical wipe. Then, the sample was pipetted in and out 3 times to thoroughly mix the sample with any residual water. Then, the cuvette was inserted into the instrument and measured. For native protein, a full wavelength spectrum (210-260nm, 1nm steps) was obtained at every concentration of guanidine, using 3s averaging time and 5 scans. For conjugates, the polymer contributes significant noise in wavelengths measured, requiring 10 scans with 5s averaging times to obtain acceptable signal/noise. Due to time constraints, a smaller wavelength window (215-230nm, 1nm steps) was collected.

Trypsin Digestion of DP5 DMAm conjugate. To a 150uL PCR tube was added 3.75uL conjugate, 3.24ul 10mM phosphate buffer, and 2uL water. The tube was heated to 95^{o}C for 2 minutes and then allowed to cool to room temperature. 1uL of Pierce Trypsin protease (Thermo product # 90057) was added (such that a 1:15 trypsin:Ub weight ratio is achieved), the tube was mixed and incubated at 37^{o}C for 20h. Upon completion, the sample was placed on ice and used for MALDI-TOF-MS within 2 hours.

MALDI-TOF-MS of DP5 DMAm conjugate. The DP5 DMAm conjugate digestion solution was mixed in a 1:2 ratio with α-Cyano-4-hydroxycinnamic acid (CHCA) matrix and spotted on the

target plate. The MALDI was calibrated with poly-alanine standards. The mass spectrum was acquired with 1000 laser shots on 50% laser power and random-walk sampling. The DP5 DMAm polymer was acquired in a similar manner. The obtained spectra are given in Figure S3a-b.

Discussion

Polymer Synthesis and Characterization. All the polymers were synthesized with relatively low dispersities, as determined by SEC. SEC did not provide useful M_n or M_w; the poor solubility of TMPA in DMF likely causes some chain compression, significantly increasing the elution times. However, the degrees of polymerization obtained by NMR agree with the targeted values, and the oxidized polymer (Figure S1a) does elute with the correct molecular weight due to transformation of the TMPA group into TEMPO.

Spin-label distance from protein surface. The spin label distance from the surface of the protein for the three block copolymers was estimated using the Flory-Fox equation (S2)

$$[\eta(M)]M = \varphi'\langle s^2(M)\rangle^{3/2} \quad \text{(S2)}$$

Where: M is the M_w of the first block of the copolymer; $[\eta(M)]$ is the intrinsic viscosity of the polymer of weight M; φ' is the Flory-Fox constant for water (3.67×10^{24}); and $\langle s^2(M)\rangle$ is the root-mean-square radius of gyration of the polymer of molecular weight M. $[\eta(M)]$ was calculated using the Mark-Houwink-Sakurada equation (equation S3), with M given by equation S4.

$$[\eta(M)] = KM^{\alpha} \quad \text{(S3)}$$

Where K and α are the empirically determined Mark-Houwink parameters for DMAm in water: 0.00232 and 0.81, respectively.[7]

$$M = (M_w/M_{n\text{-}SEC}) \times (M_{n,NMR}) \quad \text{(S4)}$$

Where $M_w/M_{n,SEC}$ is the dispersity determined by SEC; and $M_{n,NMR}$ is the number-average molecular weight determined by NMR chain-length analysis.

The Flory-Fox equation relates radius of gyration ($\langle s^2(M)\rangle^{1/2}$) and intrinsic viscosity $[\eta(M)]$ and molecular weight. Equation S5 converts the radius of gyration ($\langle s^2(M)\rangle^{1/2}$) to the root mean square end-to-end distance($\langle r^2(M)\rangle^{1/2}$).

$$\langle r^2(M)\rangle^{1/2} = 6^{1/2}\langle s^2(M)\rangle^{1/2} \quad \text{(S5)}$$

Where r is the root mean square end-to-end distance. When the first block is considered, then the mean square end-to-end distance, $\langle r^2(M)\rangle^{1/2}$, corresponds to the distance to the spin label:

$$r_{TEMPO} = \langle r^2(M_w)\rangle^{1/2} \quad \text{(S6)}$$

Where M_w is the weight averaged molecular weight of the first block copolymer.

To account for the variation in molecular weights caused by the molar mass dispersity (M_w/M_n) the relationship between the molar mass dispersity, the standard deviation of the distribution (σ_M), and mean (μ), with the mean being M_n. These parameters are related by Equation S7:

$$M_w/M_n = 1 + \frac{\sigma_M^2}{\mu^2} \quad \text{(S7)}$$

This enables the standard deviation (σ_M) of each block copolymer's molecular weight distribution to be calculated. Substituting the molecular weight $M=M_w+\sigma_M$ into equations S2-S5, where M_w is the weight averaged molecular weight of the first block and σ_M is the standard deviation of the first block, gives the distance from the protein to the spin label for a polymer of molecular weight of $M_w+\sigma_M$, or one standard deviation above M_w. Similarly, the distance to the spin label can be calculated based on $M=M_w-\sigma_M$, to give the mean distance from the protein's surface to the spin label, for a polymer of molecular weight of $M_w-\sigma_M$, or one standard deviation below the M_w. The standard variability in the position in the spin label (σ_r) due to the polymer's molar mass dispersity is estimated as shown in Equation S8:

$$\sigma_r = max\left[\left|r_{TEMPO} - \langle r^2(M_w + \sigma_M)\rangle^{1/2}\right|, \left|r_{TEMPO} - \langle r^2(M_w - \sigma_M)\rangle^{1/2}\right|\right] \quad \text{(S8)}$$

Table S1. Targeted and experimental molecular weight parameters of (co)polymers.

	Block (*composition*)	$M_{n,th}$	$M_{n,SEC}$	$M_w/M_{n,\ SEC}$	$DP_{DMAm,\ NMR}$	$DP_{TMPA,\ NMR}$
0-block	1 (*34 DMAm*)	3600	2700	1.10	37	-
	1 (*5 DMAm*)	710	620	1.12	5.8	-
A-block	2 (*4 TMPA*)	1500	490	1.20	-	4.2
	3 (*25 DMAm*)	4000	1600	1.32	36	3.9
	1 (*15 DMAm*)	1700	1400	1.12	17	-
B-block	2 (*4 TMPA*)	2500	870	1.25	-	4.5
	3 (*15 DMAm*)	4000	1800	1.26	34	3.6
	1 (*25 DMAm*)	2700	2500	1.12	29	-
C-block	2 (*4 TMPA*)	3500	1500	1.25	-	3.9
	3 (*5 DMAm*)	4000	2000	1.25	39	3.5

Polymer Oxidation. The oxidation step shown in Scheme 1 is light sensitive, and also results in oxidation of the trithiocarbonate group at the ω terminus (yellow), although the products are not well-known. The end-group is not completely oxidized, as it is still observable by UV-Vis after oxidation (and by NMR if the spin label is allowed to reduce completely). In the course of our experiments, the trithiocarbonate oxidation product is inconsequential, so we suspect it is converted into a dithiocarbonate, which is supported by a similar oxidation which was reported in 2002.[8] We believe the oxidation and cleanup conditions do not allow for significant hydrolysis of the formed dithiocarbonate to occur.[9] SEC of the oxidized polymer (Figure S1a) shows no significant perturbations in molecular weight or peak shape, indicating that no disulfide bonds are formed, and there is no mechanistic pathway where a carboxylate group would form on this end of the polymer.

SEC of oxidized polymer. C-block which had been oxidized and purified in the usual manner was analyzed by SEC to confirm that no size-altering reactions such as disulfide bonds had occurred (Figure S1a). The polymer is of the correct size and has low dispersity, with no high-MW shoulder visible – indicating that no disulfides have been formed.

EPR Spectrum of Oxidized polymer. The EPR spectra of oxidized polymer and a TEMPO standard are shown in Figure S1b. The normal hyperfine couplings are seen, but the polymer (red) is significantly broader than the standard (blue). The degree of broadening is illustrated by the fact that by integration, the polymer sample is found to have a TEMPO concentration of ca. 2mM, which is an order of magnitude higher than the concentration of the standard (0.1mM), yet the peak heights are similar, and this effect is independent of dilution. Either the polymers are restricting the spin label motion significantly, or there is a fair degree of spin-exchange coupling occurring.[2]

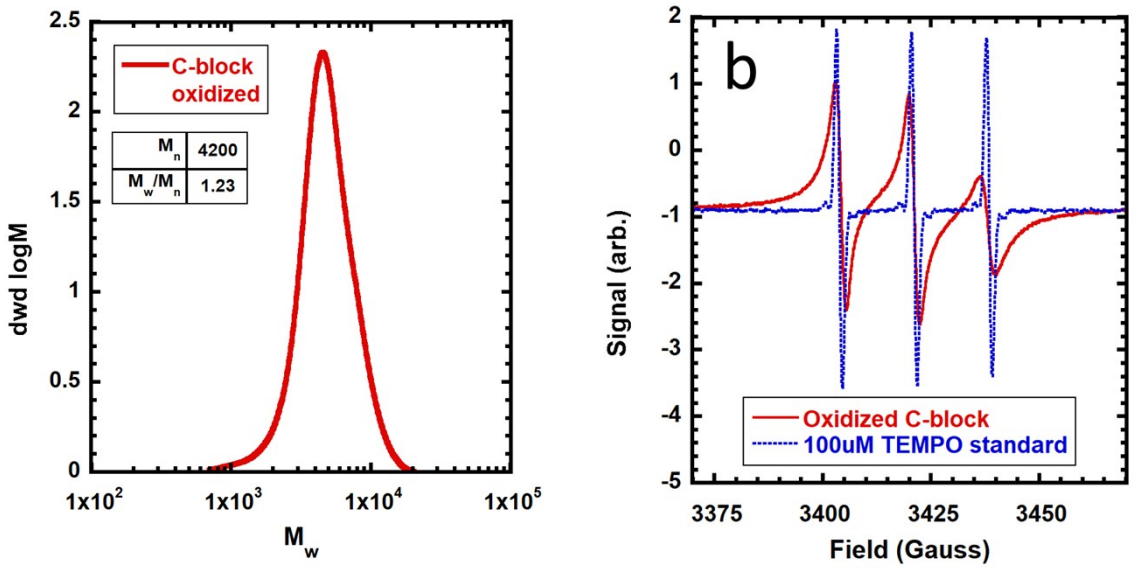

Figure S1. (a) SEC trace and molecular weight data (inset) of C-block copolymer after oxidation and cleanup. (b) Example EPR spectrum of oxidized and purified copolymer (C-block, *red*) compared to TEMPO standard (*blue*).

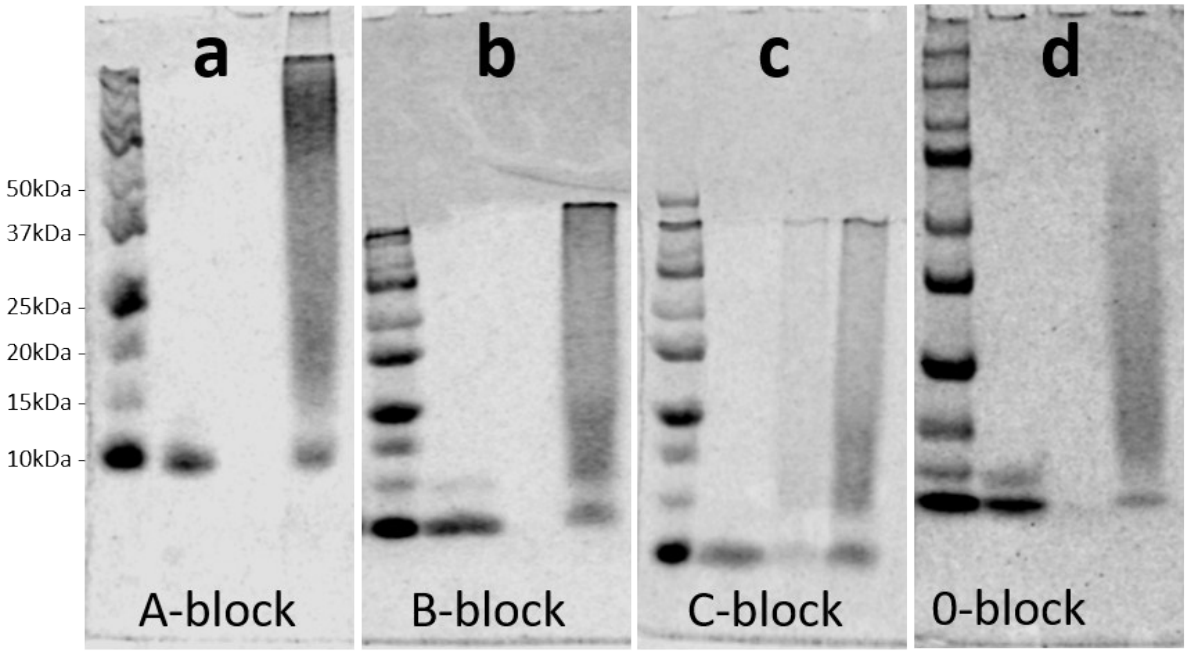

Figure S2. Conjugation efficiency of ubiquitin with (co)polymers, visualized on Coomassie-stained SDS-PAGE. (a) A-block conjugate; (b) B-block conjugate; (c) C-block conjugate; (d) 0-block conjugate. For all gels, the lanes are as follows: lane 1 – Precision Plus Protein Kaleidoscope ladder; lane 2 – native ubiquitin (1ug); lane 4 – conjugate (4ug).

Conjugation efficiency. The conjugation efficiency can be seen in Figure S2a-d. While some native protein is visible in each sample, we estimate that over 90% efficiency is achieved in every case due to the intensity of the wide conjugate bands. While 100% efficiency could be achieved, in those cases, very large conjugates that failed to enter the resolution layer of the PAGE gel were dominant species (Figure S3). We deemed these unsuitable for our NMR application due to linewidth broadening from increased size, as well as total signal loss due to PRE occurring on all faces of the protein (Figure S6).

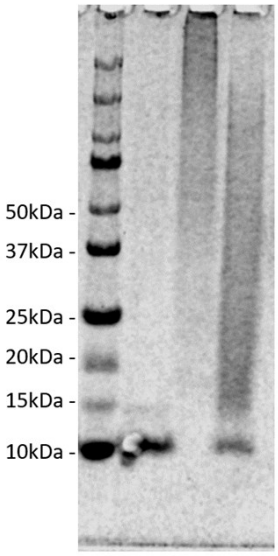

Figure S3. Conjugation of C-block to native ubiquitin with varying amounts of EDC. Lane 1 – Ladder; Lane 2 – Native Ubiquitin; Lane 3 – conjugate with 40eq C-block, 240eq EDC; Lane 4 – conjugate with 40eq C-block, 80eq EDC.

Table S2. Determination of conjugation sites by MALDI-TOF-MS

Central Mass (MALDI)[a]	Isotopic Pattern[b]	Predicted Mass	Residues (Lysines)	Polymer	Ion
667.508	Nat. Abun.	667.312	Polymer	DP4 DMAm (VA-044 α-end)[d]	Na^+
1185.627	Has M-1	1185.566	7-11 (11)	DP5 DMAm with one AA[e]	H^+
		1185.501	1-6 (N, 6)	DP2 DMAm	Na^+
1223.743	Has M-1	1223.522	7-11 (11)	DP5 DMAm with one AA[e]	K^+
		1223.662	Polymer	DP10 DMAm	Na^+
1239.721	Nat. Abun.	1239.742	Polymer	DP10 DMAm (VA-044 α-end)[d]	H^+
		1239.563	5-11 (6,11)[c]	DP3 DMAm with one AA[e]	Na^+
1575.814	Has M-1	1575.711	46-54 (48)[c]	DP4 DMAm	H^+
1717.023	Has M-1	No match			
2051.103	Has M-1	2051.001	43-54 (48)	DP5 DMAm	H^+
		2050.992	55-63 (63)	DP8 DMAm with one AA[e]	H^+
2132.095	Has M-1	2131.967	30-42 (33)	DP4 DMAm	H^+
2843.548	Has M-1	2843.391	55-72 (63)	DP5 DMAm	H^+

[a] Highest intensity peak in a series of +/- 99amu.

[b] Because 99% ^{15}N Ub was used, peptide-containing peaks will have a small peak at exactly 1amu less than the parent ion resulting from the 1% ^{14}N, while peaks not resulting from peptides will have no such signature (Figure S4b)

[c] Peptide resulting from trace chymotrypsin contamination (cleavage at phenylalanine).[10]

[d] Polymer chains initiated by VA-044 rather than RAFT-CTA (do not conjugate)

[e] Acrylic acid (AA) can result from hydrolysis of DMAm in aqueous solution.

MALDI-TOF-MS Determination of Conjugation Sites. Table S2 and Figure S4b show the peaks resulting from trypsin digestion of a short DMAm-Ub conjugate. A short polymer, rather than a small molecule, was chosen in order to better represent the bulk and polarity of the larger polymers – where exposed surface area of a reactive amine is a critical parameter. Many potential matches were found, and several observed peaks have two potential matches. In some cases, both matches may actually be present as evidenced by the peaks in the spectrum having two crests rather than just one; however little stock is placed in this possibility due to the poor signal/noise for some of these peaks.

It is notable that several matches correspond to polymers with the presence of an acrylic acid resulting from hydrolysis of DMAm. This is plausible due to the extended incubation period (20-24h) required to fully digest Ub, which is quite resistant to proteolysis. Two matches also result from off-target cleavages, a documented occurrence even in commercial trypsin

formulations.[10] These are observable due to the extended incubation time and increased trypsin concentration (we use 15:1 Ub:trypsin whereas 20-100 is recommended but was insufficient).

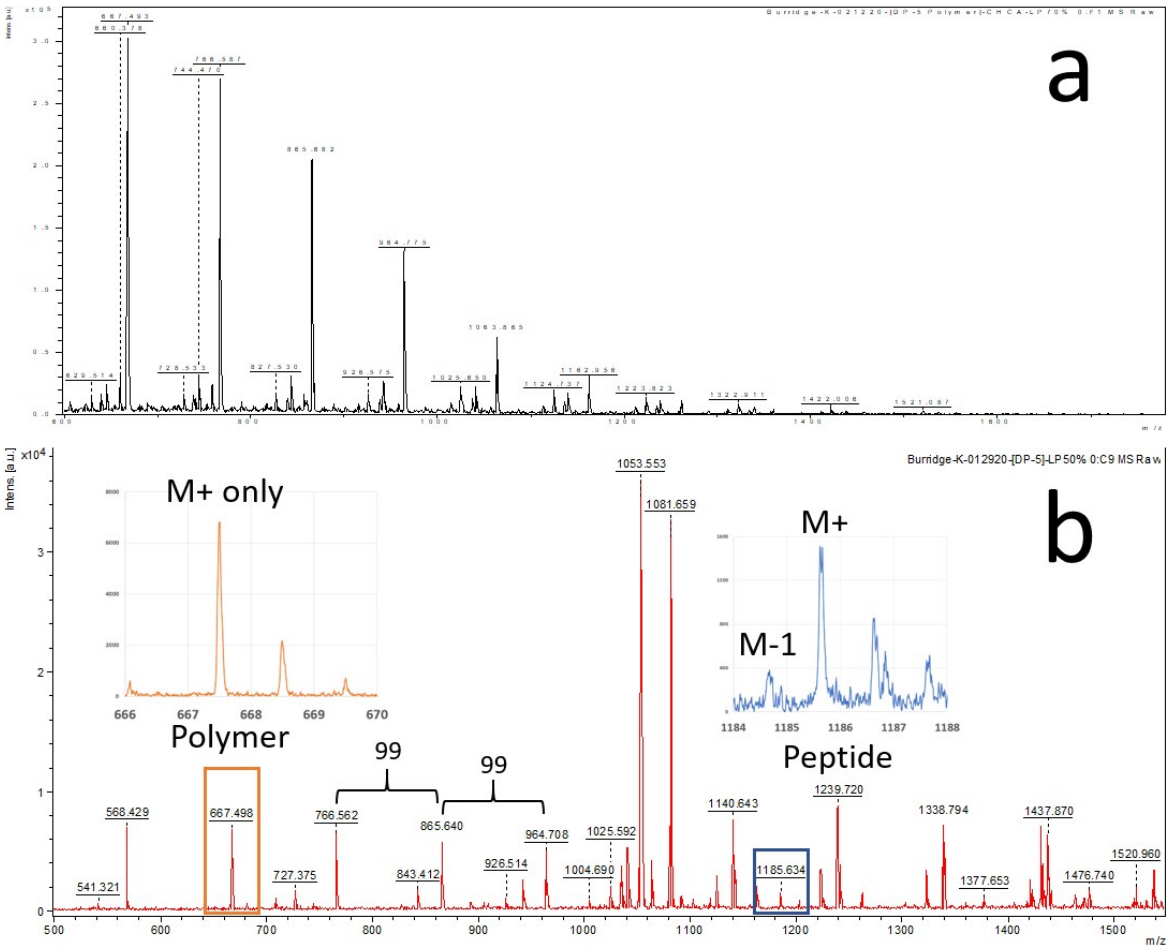

Figure S4. MALDI-TOF-MS spectra of (a) DP5 DMAm polymer and (b) digested DP5 DMAm conjugate, with insets showing isotopic signatures of polymer versus peptide peaks.

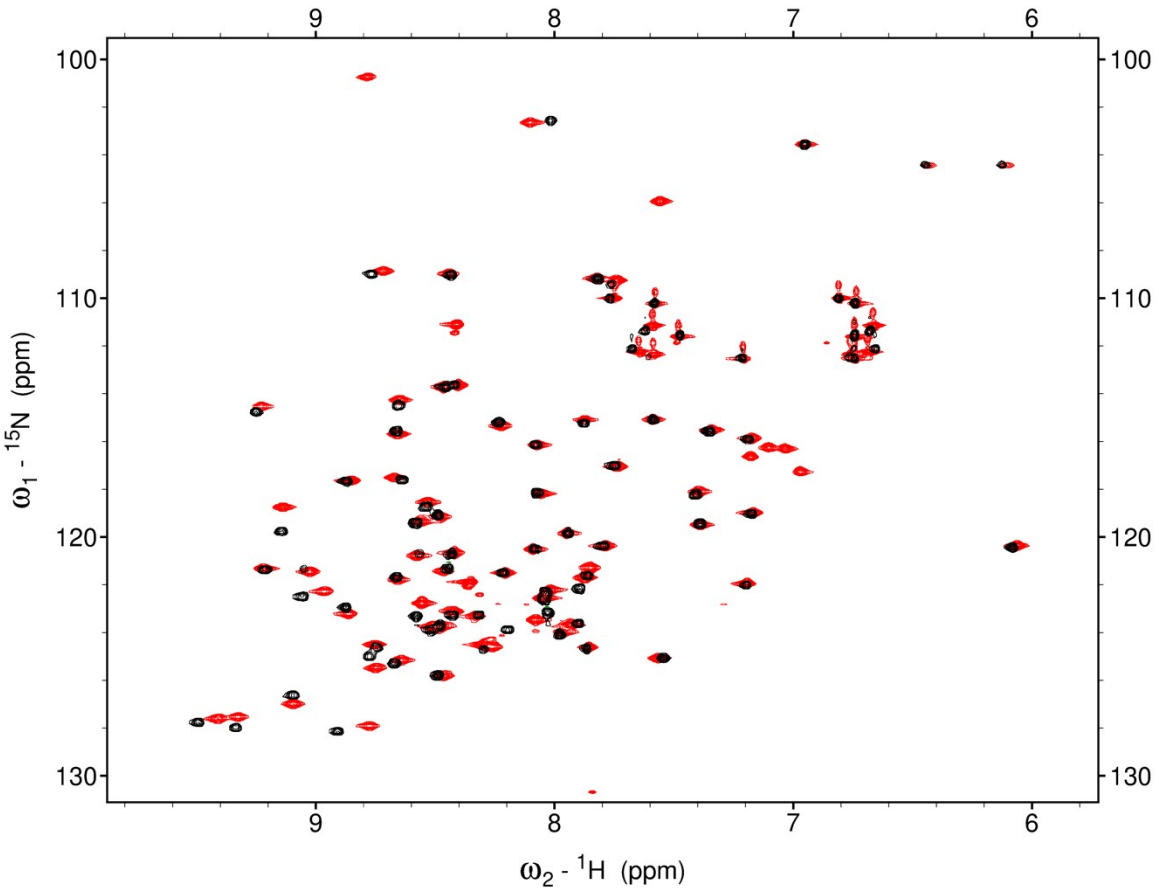

Figure S5. ^{1}H/^{15}N-HSQC of native ubiquitin (black) in pure aqueous media, and in the presence of 40 equivalents of spin labeled B-block polymer (red)

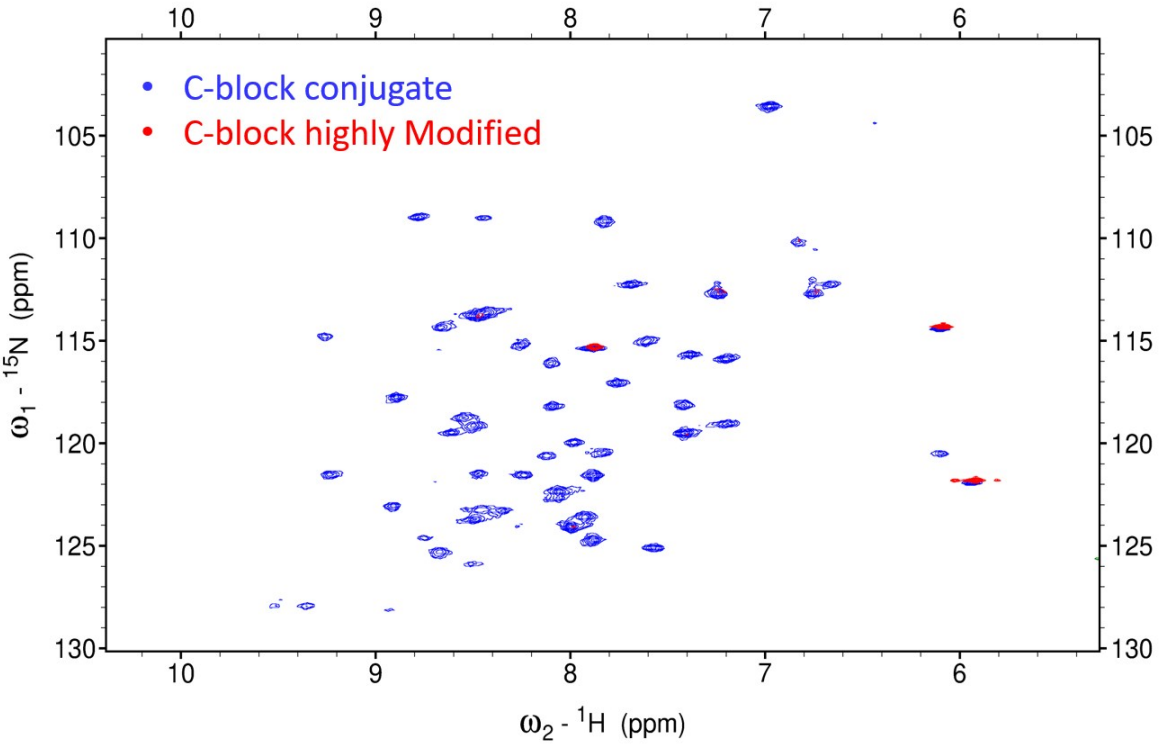

Figure S6. $^{1}H/^{15}N$-HSQC of C-block conjugates; 2-3 polymer chains (*blue*); 4-6 polymer chains (*red*)

References

[1] T. A. Wright, M. Lucius Dougherty, B. Schmitz, K. M. Burridge, K. Makaroff, J. M. Stewart, H. D. Fischesser, J. T. Shepherd, J. A. Berberich, D. Konkolewicz, et al., *Bioconjug. Chem.* **2017**, *28*, 2638-2645

[2] N. Chikushi, H. Yamada, K. Oyaizu, H. Nishide, *Sci. China Chem.* **2012**, *55*, 822–829.

[3] F. Delaglio, S. Grzesiek, G. W. Vuister, G. Zhu, J. Pfeifer, A. Bax, *J. Biomol. NMR* **1995**, *6*, 277–293.

[4] W. Lee, M. Tonelli, J. L. Markley, *Bioinformatics* **2015**, *31*, 1325–7.

[5] V. A. Jaravine, A. V. Zhuravleva, P. Permi, I. Ibraghimov, V. Y. Orekhov, *J. Am. Chem. Soc.* **2008**, *130*, 3927–3936.

[6] B. Ibarra-Molero, V. V. Loladze, G. I. Makhatadze, J. M. Sanchez-Ruiz, *Biochemistry* **1999**, *38*, 8138–8149.

[7] F. Asmussen, K. Ueberreiter, *J. Polym. Sci.* **1962**, *57*, 199–208.

[8] P. Vana, L. Albertin, L. Barner, T. P. Davis, C. Barner-Kowollik, *J. Polym. Sci. Part A Polym. Chem.* **2002**, *40*, 4032–4037.

[9] P. J. Bracher, P. W. Snyder, B. R. Bohall, G. M. Whitesides, *Orig. Life Evol. Biosph.* **2011**, *41*, 399–412.

[10] P. Fang, M. Liu, Y. Xue, J. Yao, Y. Zhang, H. Shen, P. Yang, *Analyst* **2015**, *140*, 7613–7621.