

Supplementary Information for “The Best of Both Worlds: Active Enzymes by Grafting-To followed by Grafting-From a Protein”

Rebecca Falatach, Cameron McGlone, M. Sameer Al-Abdul-Wahid, Saadyah Averick, Richard C. Page, Jason A. Berberich, Dominik Konkolewicz

Experimental

Materials. Hen Egg White Lysozyme was purchased from MP Biomedicals. Acrylamide and hydrochloric acid were obtained from Fisher Scientific. *Micrococcus lysodeikticus*, 4-methylumbelliferyl β -D-N,N',N''-triacetylchitotrioside, azobisisobutyronitrile (AIBN), ethanethiol, carbon disulfide, 2-bromopropionic acid, and oligo(ethylene oxide) methyl ether acrylate (OEOA) were purchased from Sigma Aldrich. Deuterated DMSO was obtained from Acros Organics. N-hydroxysuccinimide and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide were purchased from Thermo Scientific. Sodium hydroxide was obtained from Ricca chemicals, and sodium carbonate was obtained from Tedia. VA-044 was obtained from Wako chemicals. All materials were used without further purification. SDS-poly(acrylamide) gel electrophoresis was performed using Bio-Rad Mini-PROTEAN TGX 4-20% gradient gels. Gels were stained with GelCode Blue protein stain obtained from Thermo Scientific.

¹H Nuclear Magnetic Resonance. ¹H NMR spectra were recorded on a Bruker 300 MHz NMR spectrometer equipped with a broadband inverse probe. A standard pulse-acquire sequence was used with a 30° tip angle (*zg30*).

Matrix Assisted Laser Desorption/Ionization-Mass Spectrometry (MALDI-MS). MALDI-TOF spectra were collected on a Bruker Autoflex III MALDI-TOF mass spectrometer (Billerica, MA). Mass spectra were calibrated using Bruker Protein Calibration Standard I (spanning 5.7 kD to 16.9 kD) as an external standard. Generally, 0.5 μ L of sample (0.5 mg/ml) was mixed with 0.5 μ L of saturated sinapinic acid (Fluka, WI) solution (0.1 % TFA, 40% acetonitrile) directly on the target plate and allowed to dry at room temperature. Samples were analysed in the positive ion linear mode to detect [M + H]⁺ ions.

Electrospray Ionization-Mass Spectrometry (ESI-MS). Direct injection ESI-MS spectra were collected on a Bruker Esquire-LC mass spectrometer (Billerica, MA) operating in negative ion mode. Samples were injected at a rate of 7 μ L / minute, with a nebulization gas pressure of 7 psi and a heated nitrogen drying gas flow rate of 4 L / minute at a temperature of 300 °C. The capillary voltage was set to 4000 V, and ions were allowed to accumulate in the trap for 4-10 ms. The nominal target mass was optimized for each sample and found to be 750 m/z for DP5, 1100 m/z for DP10, and 1700 m/z for DP20. Up to 200 scans, spanning 50 – 2200 m/z, were collected in order to obtain sufficient signal-to-noise.

Synthesis of 2-(((ethylthio)carbonothioyl)thio)propanoic acid (PAETC).

Sodium hydroxide (20.5g, 0.51 mol) was dissolved in 200 mL of water. Ethanethiol (42 mL, 36.1 g, 0.58 mol) was added dropwise, and allowed to react for 20 min, after which point carbon disulfide (40 mL,

61.6g, 0.81 mol) was added dropwise over approximately 10 min. The reaction mixture was stirred at room temperature for 40 min, affording a dark orange solution. The aqueous solution was washed with 4 x 100 mL of dichloromethane, and the solvent was removed to afford an orange solid of sodium ethyltrithiocarbonate. To a clean round bottom flask sodium ethyltrithiocarbonate (48g, 0.30 mol) was added. To this flask acetone (150 mL) was added followed by the dropwise addition of 2-bromopropionic acid (43 g, 0.30 mol). The reaction mixture was allowed to stir overnight giving a yellow solution. The solid of sodium bromide was removed by vacuum filtration, and the solvent removed under reduced pressure. The product was redissolved in acetone and a white product was recrystallized from the yellow solution. The acetone was removed under reduced pressure, and finally the product was purified by dissolving the product in diethyl ether (150 mL), and washed with saturated sodium carbonate (150 mL). The ether layer was discarded and the aqueous layer was washed with 4 x 100 mL of diethyl ether. A final layer (100 mL) of ether was added, and the aqueous layer was acidified with concentrated hydrochloric acid. The ether layer was washed once with water (50 mL) and the ether layer was collected. The solvent was removed under reduced pressure to afford 34 g (53% isolated yield) 2-(((ethylthio)carbonothioyl)thio)propanoic acid or (propionic acid)ylethyl trithiocarbonate (PAETC). The purity was confirmed by NMR and was found to be greater than 97%. ¹H-NMR (300 MHz, CDCl₃) δ ppm 1.63 (3H, d, *J* = 7.4 Hz, CH₃CH(S)COOH) 4.87 (1H, q, *J* = 7.4 Hz, CH₃CH(S)COOH), 3.38 (2H, q, *J* = 7.4 Hz, CH₃CH₂S), 1.36 (3H, t, *J* = 7.4 Hz, CH₃CH₂S).

Conjugation of PAETC to Lysozyme. For NHS/EDC coupling reactions, lysozyme, CTA, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC), and N-hydroxysulfosuccinimide (sulfo-NHS) were combined in 0.1M phosphate buffer at pH 7.4 in a flask and allowed to react at room temperature for 2hrs before being quenched by glycine. Lysozyme was always added at a final concentration of 1mg/ml, sulfo-NHS was always kept at a final concentration 5mM, and EDC was added at a molar ratio of 1 EDC to 1 CTA. A sample reaction is as follows: Lysozyme (5mg, 0.35μmol), CTA (25.73mg, 0.122mmol), EDC (23.46mg, 0.122mmol), and NHS (5.429mg, 25μmol) were added to the reaction vial using stock solutions all prepared in the reaction buffer. The molar ratio of CTA to amines on the lysozyme (6 lysine residues plus 1 terminal amine) was varied to determine optimal conditions. Higher ratios of CTA to amine resulted in higher modification as determined by MALDI, although the solubility was lower. This conjugated protein was washed with 20mM MES buffer at pH 6 using an Amicon stirred ultrafiltration cell with a 10kDa MWCO membrane to remove excess CTA, EDC, and NHS until the absorbance at 309nm of the solution inside the cell held constant.

UV-Vis Spectroscopy. A Spectronic Genesys 5 spectrophotometer was used to collect UV-Vis spectrums. UV-Vis spectroscopy was used to determine the approximate molar ratio of CTA attached to lysozyme. A calibration curve was developed to determine that the extinction coefficient of the PAETC at 309nm is 11700 M⁻¹cm⁻¹. Once the conjugated lysozyme was washed using the stirred ultrafiltration cell the absorbance spectrum was recorded and the concentration of the protein determined by the peak at 280nm was compared to the concentration of the CTA determined by the peak at 309nm to get an approximate ratio of CTA to lysozyme.

Lysozyme Activity Assays. Two different sized substrates were used to determine the activity of the protein at each stage of modification. The large substrate was *Micrococcus lysodeikticus*, a common bacteria used to test the activity of lysozyme. The reaction between lysozyme and this bacteria's cell wall

can be monitored by the change in absorbance at 450nm over time. The reaction was done in a 66mM KH_2PO_4 buffer at pH 6.2. This assay was done using a BioTek Synergy HT plate reader and used the kinetic feature to measure the absorbance every 15 seconds over a 4 minute time period. The slope of the line produced from graphing absorbance vs time represented the rate of reaction and when compared to the rate of reaction of a native lysozyme control the percent activity retention could be determined. The smaller substrate used was 4-methylumbelliferyl β -D-N,N',N''-triacetylchitotrioside ((NAG)₃-MUF), which when hydrolysed by the lysozyme produces a fluorescent product. This reaction was monitored using the fluorescence function on the same plate reader using an excitation/emission setting of 360/460. This reaction took place in a 50mM Acetate buffer at a pH of 5.5, but the fluorescent product fluoresces at a higher pH so the reaction was stopped using glycine buffer at a pH of 10.8 before measuring the fluorescence. This assay also requires time point measurements but the reaction takes place over an hour, so time points were taken at time 0, 30 minutes and 60 minutes. In between measurements, the plate was kept at 37°C and in a dark place to reduce exposure of the substrate to light.

RAFT polymerization of Acrylamide with CTA. To synthesize the oligoCTA, RAFT polymerization was used to grow a short oligomer of acrylamide from the CTA in a 50:50 mixture of methanol and water. Dimethylformamide (DMF) was added at 4% of the volume strictly for use in conversion determination using ¹H NMR. Molar ratio for CTA to AIBN was 1 to 0.1. The molar ratio of CTA to Am was adjusted based on desired length of oligomer (i.e. DP=5 had a molar ratio of CTA to Am of 1 to 5). The reaction conditions used to produce the DP=5 oligoCTA used in the OEOA chain extension was as follows: CTA (1.99g, 9.2mmol), acrylamide (3.389g, 47.6mmol), AIBN (0.156g, 0.95mmol) and 0.5ml of DMF were added to a round bottom flask with 12.6ml of the methanol/water mixture. This reaction was purged with nitrogen for 5 minutes to remove oxygen, then placed in an oil bath at 65°C for 21 hrs. The conversion was determined using ¹H NMR. These oligoCTAs were precipitated by adding the reaction mixture dropwise to tetrahydrofuran (THF) that was cooled in an ice bath. The precipitated oligoCTA was vacuum filtered and rinsed into a pre-weighed round bottom flask. The solvent was then removed under reduced pressure. ESI-MS was used to determine the average molecular weight of the oligoCTA and the distribution of oligomer lengths and ¹H NMR in DMSO-*d*₆ was used to determine the monomer conversion.

Conjugation of oligoCTA to lysozyme. The conjugation of the oligoCTA to lysozyme was done using the same NHS/EDC coupling method as previously mentioned. However, the efficiency greatly decreased with an increase in the oligomer chain length so the molar ratio of oligoCTA to amine was significantly increased to overcome this. The conditions used to conjugate the DP=5 oligoCTA to lysozyme for use in the chain extension reaction are as follows: Lysozyme (100mg, 7 μ mol), oligoCTA (3.3g, 5.87mmol), EDC (1.126g, 5.87mmol), and NHS (57.5mg, 0.5mmol) were added to the reaction flask at a final volume of 100ml of the 0.1M phosphate buffer at pH 7.4. These conjugates were purified by dialysis using an Amicon stirred ultrafiltration cell dialysis unit and were washed with 20mM MES buffer at pH 6 until the absorbance at 309nm of the solution inside the cell held constant.

RAFT polymerization of OEOA from oligoCTA-Lysozyme conjugate. OEOA, oligoCTA-Lysozyme conjugate, and VA-044 were combined in a 5 ml round bottom flask. The conjugated lysozyme, in the 20mM MES buffer at pH6, was concentrated to 10 mg/ml. OEOA (45.25 μ l, 49.318mg, 102.75 μ mol) and

VA-044 (0.831mg, 2.569 μ mol) were added to 400 μ l of the concentrated oligoCTA-lysozyme conjugate. This solution was degassed for 5 minutes with nitrogen and sealed to remove all oxygen. It was then heated in an oil bath set at 35°C for 21 hours. ¹H NMR was used to determine that the monomer conversion went to over 90%. The electrophoresis gel shown in Figure 3 shows that very little conjugated lysozyme was left unmodified.

SDS-PAGE for Native, oligoCTA- and OEQA-Lysozyme conjugates. Samples containing 7.8 μ g each of native, oligoCTA- and OEQA-Lysozyme conjugate samples were mixed with 4x LDS loading buffer containing 50mM DTT. Samples were incubated at 70°C for 10 minutes prior to loading on a TGX 4-20% gradient polyacrylamide gel. Protein bands stained with GelCode Blue protein stain were visualized by fluorescence at 700nm using a LI-COR Odyssey Fc imager.

Additional Figures and Characterization data.

Characterization of the CTA-lysozyme conjugate

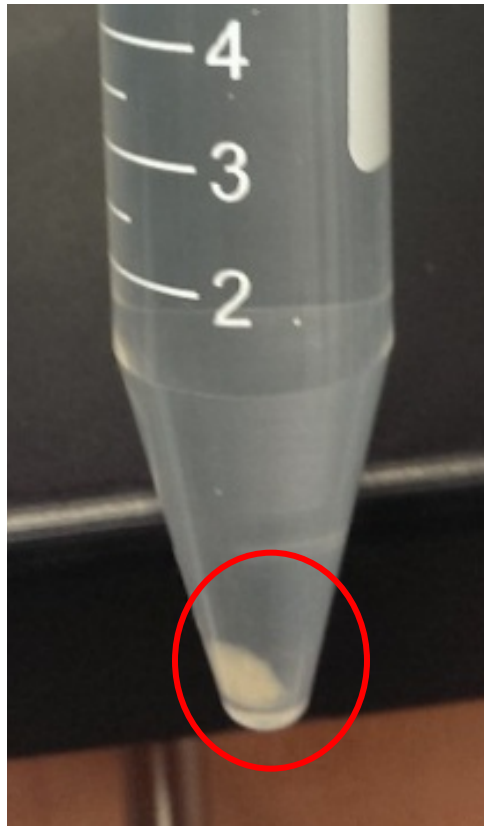


Figure S1. Protein-CTA conjugate precipitated out of solution due to high modification and therefore a loss of solubility. Conjugation performed under the conditions: Lysozyme (5mg, 0.35 μ mol), CTA (25.73mg, 0.122mmol), EDC (23.46mg, 0.122mmol), and NHS (5.429mg, 25 μ mol), with Lysozyme at 1 mg/mL.

The ratio of the peak at 309 nm to the peak at 280 nm was used to approximate the average number of CTA attachments on the lysozyme. These ratios were then compared to the MALDI-MS spectrums. Figure S2 shows the absorbance spectrum of native lysozyme compared to the CTA-lysozyme conjugate shown in Figure S1. The reduced peak at 280nm shows that the precipitate in Figure S1 is protein. The shoulder at 309nm is due to the CTA from which the ratio of CTA to lysozyme was determined to be about 2, which represents an average of 2 CTA attachments per lysozyme. This method, however, could not be used for the oligoCTAs because the broad peak at 309nm obscures the protein peak at 280nm making it difficult to determine an accurate protein concentration.

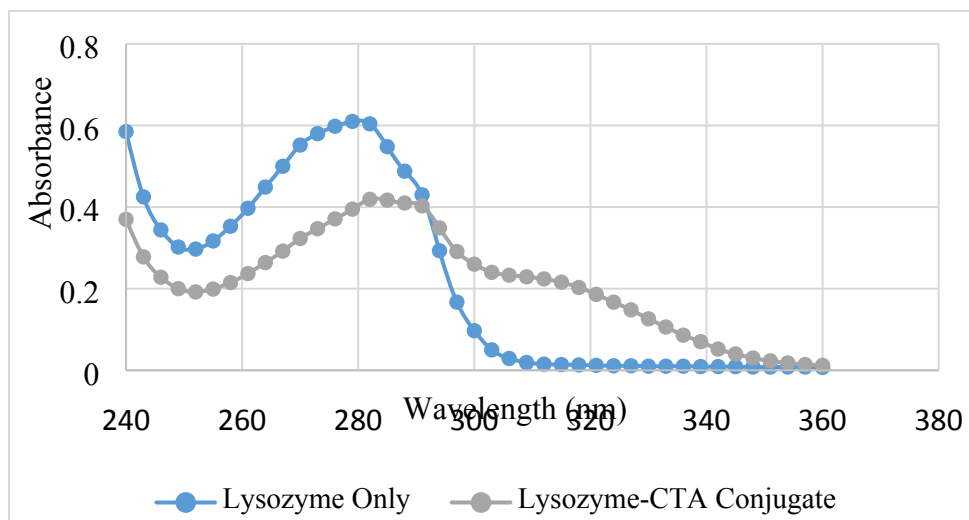


Figure S2. Absorbance spectrum of lysozyme-CTA conjugate shown in Figure S1 compared to native lysozyme.

Characterization of the oligomers that are grafted to lysozyme.

Figure S3 shows the ESI-MS results for oligoCTAs of chain lengths DP=10 and DP=20 the DP=5 oligomer in Figure 1A. Table S1 contains theoretical and experimentally determined molecular weights for these three oligoCTAs.

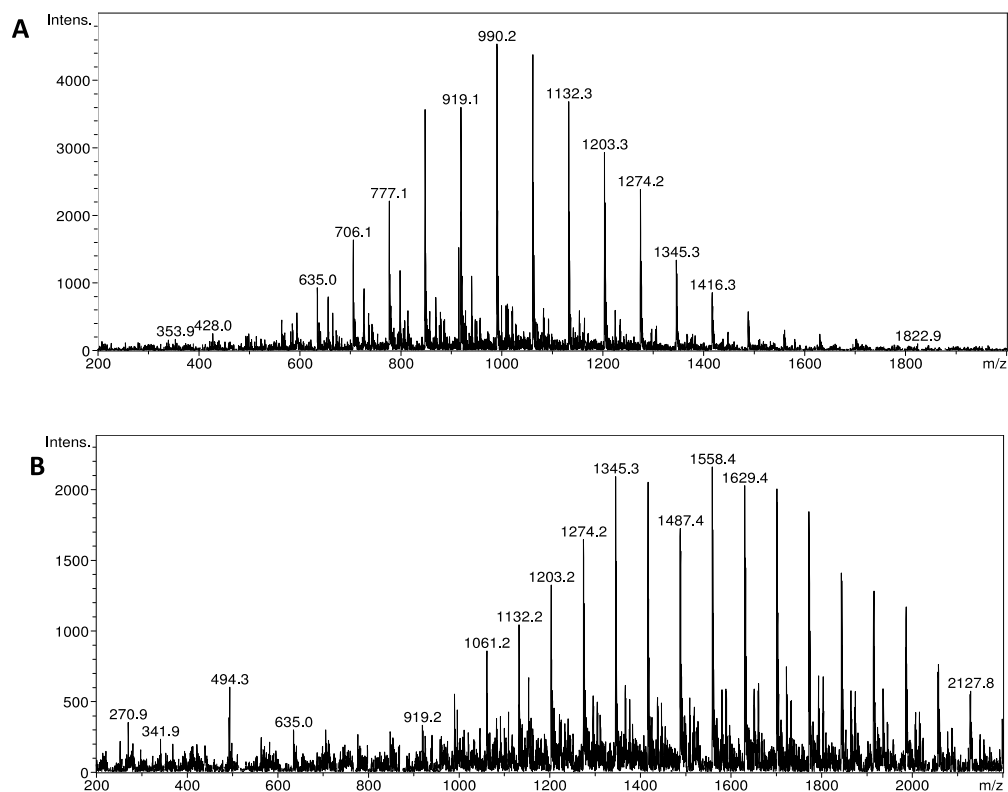


Figure S3. ESI-MS data for A the DP = 10 oligoCTA and B the DP = 20 oligoCTA.

Table S1. Estimated average degree of polymerization and dispersity for the DP = 5, 10 and 20 oligoCTA, as determined by fitting a Gaussian distribution to the ESI-MS data.

Sample	Average Degree of Polymerization (from ESI-MS) ^a	Estimated Dispersity (from ESI-MS) ^b
DP 5 Am oligoCTA	7.9	1.07
DP 10 Am oligoCTA	11.4	1.07
DP 20 Am oligoCTA	18.7	1.08

a Estimated as the mean of a Gaussian distribution fitted to the ESI-MS data.

b Estimated as $1 + \mu^2/\sigma^2$, where μ is the mean and σ is the standard deviation of a Gaussian distribution fitted to the ESI-MS data.

Additional Characterization of Protein Polymer Conjugates.

The MALDI-MS of each of the oligoCTA-lysozyme conjugates that were produced using a ratio of 120 oligoCTA per amine are shown in Figure S4

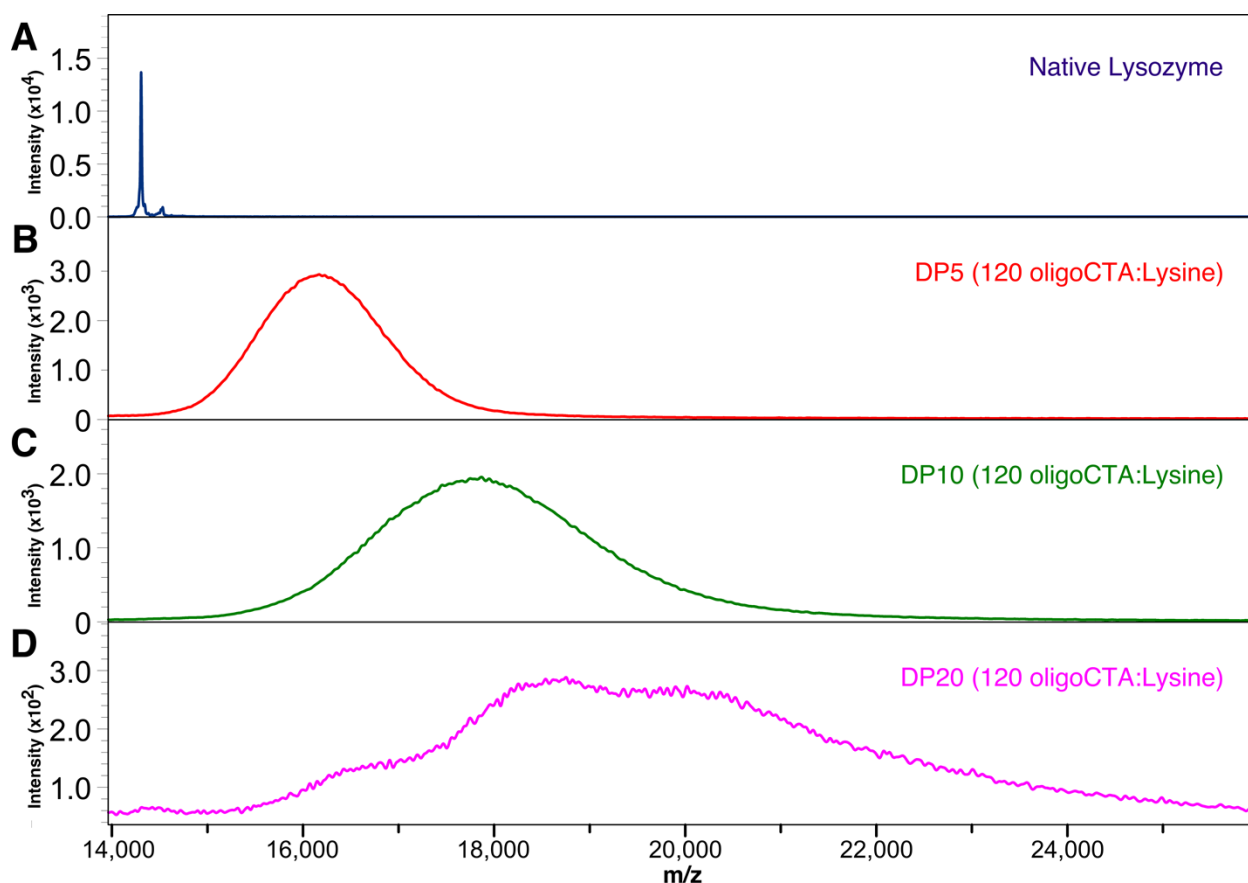


Figure S4. MALDI-MS data for lysozyme conjugated with either the DP=5, 10 or 20 oligomer with a ratio of macroCTA to amine on lysozyme of 120:1 using insitu NHS/EDC coupling.

To determine the average number of oligoCTAs per lysozyme, the theoretical molecular weight of an oligoCTA-lysozyme conjugate with one, two, and three attachments was determined and then compared to the MALDI-MS spectrum. For example, a lysozyme with one attachment would have an approximate theoretical MW of 14,300Da (lysozyme) + 210Da (CTA) + 7.9*71Da (acrylamide with DP=5 from ESI) = 15071Da. This is repeated for two, three, or even four attachments then peaks at those approximate molecular weights are marked. To determine an approximate average number of attachments, the relative intensities at each of those peaks are used to find a weighted average.