SUPPLEMENTARY DATA FOR BELLUCCI, ET AL.

SUPPLEMENTARY FIGURE LEGENDS

Supplementary Figure 1. Schematic of fusion protein constructs showing the linkers used. Sortase recognition sites are shown in bold. (a) SrtA fusion with ELP. (b) SrtA-ELP-GFP and SrtA-ELP-TNFα.

Supplementary Figure 2. SDS-PAGE analysis of TRX-ELP fusion incubated with SrtA-ELP fusion with added EGTA. 5 mM EGTA quenches the cleavage reaction, as seen by the presence of a band corresponding to intact TRX-ELP and the absence of a band corresponding to free ELP.

Supplementary Figure 3. (a) Dependence of conversion efficiency on the molar ratio of substrate to enzyme was analyzed by SDS-PAGE. The SrtA-ELP (enzyme) and TRX-ELP fusion (substrate) were run separately as controls in the first two lanes. All other lanes show the reaction product at a substrate:enzyme molar ratio specified above the lane. (b) Dependence of reaction conversion on temperature was analyzed by SDS-PAGE for reactions of SrtA-ELP and TRX-ELP at a substrate:enzyme of 4:1 (mol/mol). All reactions used an initial substrate concentration of 100 μM and were incubated for 18 hours.

Supplementary Figure 4. Inverse phase transition behavior of SrtA-ELP fusion (●), SrtA-ELP-GFP (●), and SrtA-ELP-TNFα (●) in response to heating in MilliQ water. Absorbance was measured at 300nm during a 1 ºC/min temperature ramp from 15-30°C for each fusion protein at 25 μM. The transition tempertaure is defined as the inflection point for each curve.

Supplementary Figure 5. Conditions for target protein purification from cleavage reactions were selected to ensure aggregation of TRX-ELP, which was expected to have a high Tt because TRX was one of the more hydrophilic target proteins tested and the concentration of TRX-ELP was low after the cleavage reaction. (a) Thermal response of TRX-ELP at 12.5 μM in cleavage reaction buffer with NaCl at a concentration of (●) 0M, (●) 0.25M, (●) 0.5M, (●) 0.75M, and (●) 1M. Absorbance was measured at 300nm during a 1ºC/min temperature ramp from 20-70°C. (b) The inverse transition temperature (T_t) of TRX-ELP shows a linear relationship with NaCl concentration over the range tested for fusion protein concentrations of (●) 12.5, (●) 25, and (●) 50 μM. The TRX-ELP T_t in reaction buffer was reduced approximately 20°C by the addition of 1M NaCl, which was sufficient to isothermally trigger the transition at 40°C for a fusion protein concentration as low as 10 μM.

Supplementary Figure 6. HPLC elution profiles for (a) GFP, (b) TNFα, and (c) TRAIL purified from reactions of ELP fusion proteins with SrtA-ELP. (d) Comparison of elution profiles for unreacted TRAIL-ELP (—) and recovered TRAIL product (—). All traces show the absorbance measured at 280 nm.

Supplementary Figure 7. MALDI-TOFMS mass spectrum of TRX, GFP, TNFα, and TRAIL purified from reaction of target protein-ELP fusions with SrtA-ELP. The theoretical, isotope-averaged masses are 13133.86 Da, 27874.41 Da, 18328.60 Da, and 20563.86 Da for TRX, GFP, TNFα, and TRAIL, respectively. Homodimers and homotrimers are suggested by the spectra of TNFα and TRAIL, though this is not conclusive evidence that they exist. A indicates the analyte and M signifies a matrix molecule (sinapinic acid).

Supplementary Figure 8. Native PAGE of TNFα. Lane 1: TNFα produced by ternary reaction spiked with 0.5% (m/v) bovine serum albumin (BSA). Lane 2: commercial TNFα from eBioscience supplied with 0.5% BSA as a stabilizer. Lane 3: TNFα from the ternary fusion reaction without added BSA. BSA is known to aggregate and run as a ladder of multimers in native PAGE depending on its preparation conditions and handling, which disallows exact reproduction of the banding pattern of the commercial product.

Supplementary Figure 9. Static light scattering analysis of TNFα purified from a ternary SrtA-ELP- TNFα fusion indicated an average molecular weight of 44.7 +/- 0.14 kDa.

Supplementary Figure 10. Triglycine-modified camptothecin used in conjugation reactions with TRAIL.

Supplementary Figure 11. ESI-MS of (a) TRAIL-Gly_3 and (b) TRAIL-CPT. Deconvolution of the spectra (inset of panels a and b) indicate average molecular weights of 20564.3 Da and 20896.2 Da for TRAIL-Gly_3 and TRAIL-CPT, respectively. The theoretical molecular weights are 20563.9 Da and 20893.2 Da for TRAIL-Gly_3 and TRAIL-CPT, respectively.

Supplementary Figure 12. Reverse-phase HPLC elution profiles for TRAIL-Gly_3 (—) and TRAIL-CPT (—) are shown for the 280 nm absorbance channel (a). The full elution time for TRAIL-Gly_3 (—) and TRAIL-CPT (—) is compared to the elution profile for Gly_3-CPT (—) in the 365 nm absorbance channel. 365 nm was selected to monitor CPT elution as the drug absorbs strongly at this wavelength (Supplementary Fig. 14).

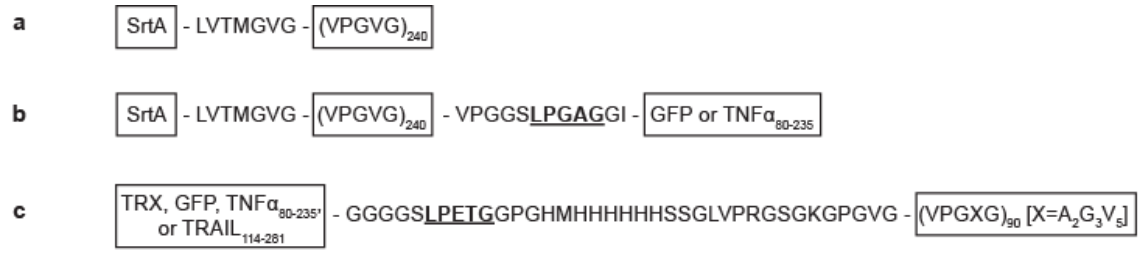
Supplementary Figure 13. (a) Calibration curve for calculating CPT concentration in water as a function of absorbance at 280 nm and 365 nm. The curves are based on serial dilutions of a CPT stock solution made by dissolving a known mass into a known volume. (b) Equations used to determine the TRAIL and CPT concentrations of the conjugation product based on the A280 and A365 of the solution. These account for the contributions of both CPT and TRAIL to the measured A280. (c) Representative absorbance spectra of TRAIL-CPT.

Supplementary Figure 14. Caspase 3 and 7 activation in MDA-MB-231 cells treated for 5 hours with the indicated concentrations of TRAIL purified from TRAIL-ELP was measured using a luminescent assay from Promega. The fold increase in luminescence is reported for each concentration relative to an untreated control group. Error bars indicate standard deviations.

Supplementary Figure 15. Figure 4d is reproduced, but the anticipated effect of adding the dose-response functions for Gly_3-CPT and TRAIL is also shown (—).

Supplementary Figure 16. Cell death in PC3 cells treated with TRAIL (●), Gly_3-CPT (●), TRAIL-CPT conjugate (●), or a mixture of Gly_3-CPT and TRAIL at a 0.8:1 molar ratio. (●). For combination treatments, the CPT concentration is indicated. The viable cell number is reported relative to an untreated group. Error bars indicate standard deviations.

SUPPLEMENTARY FIGURES



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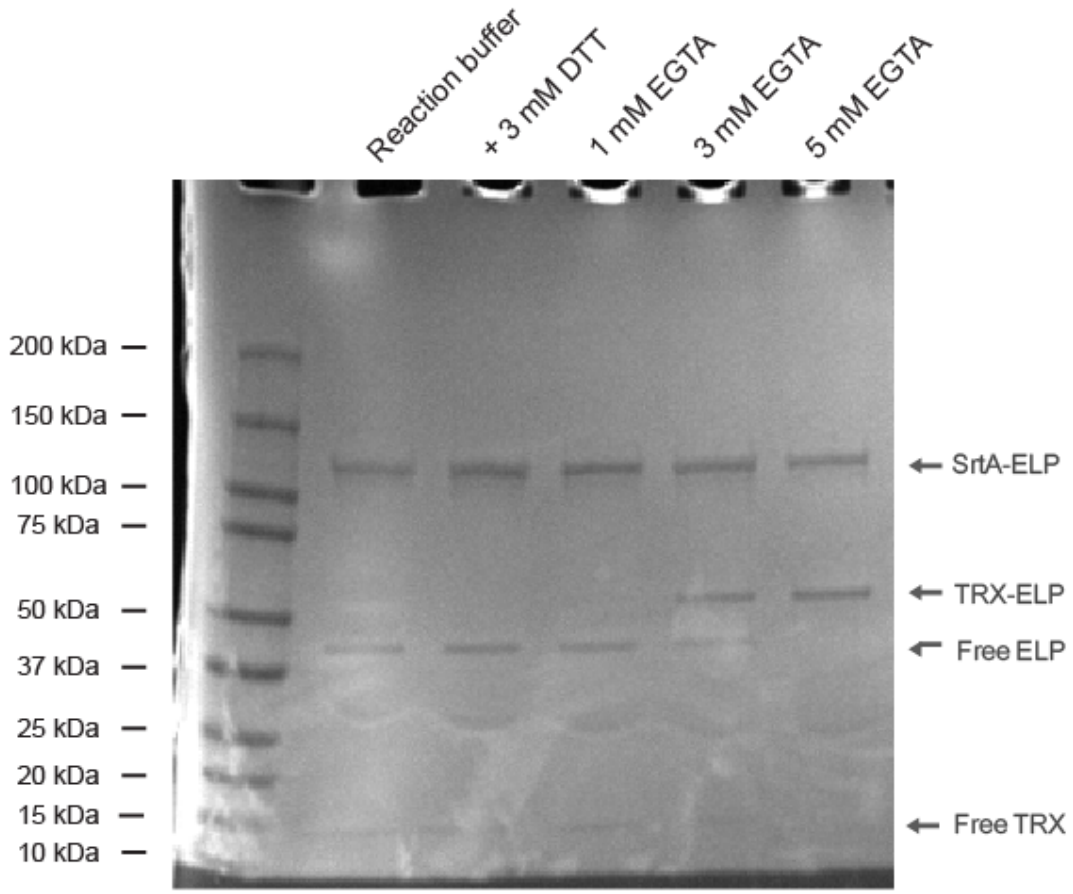

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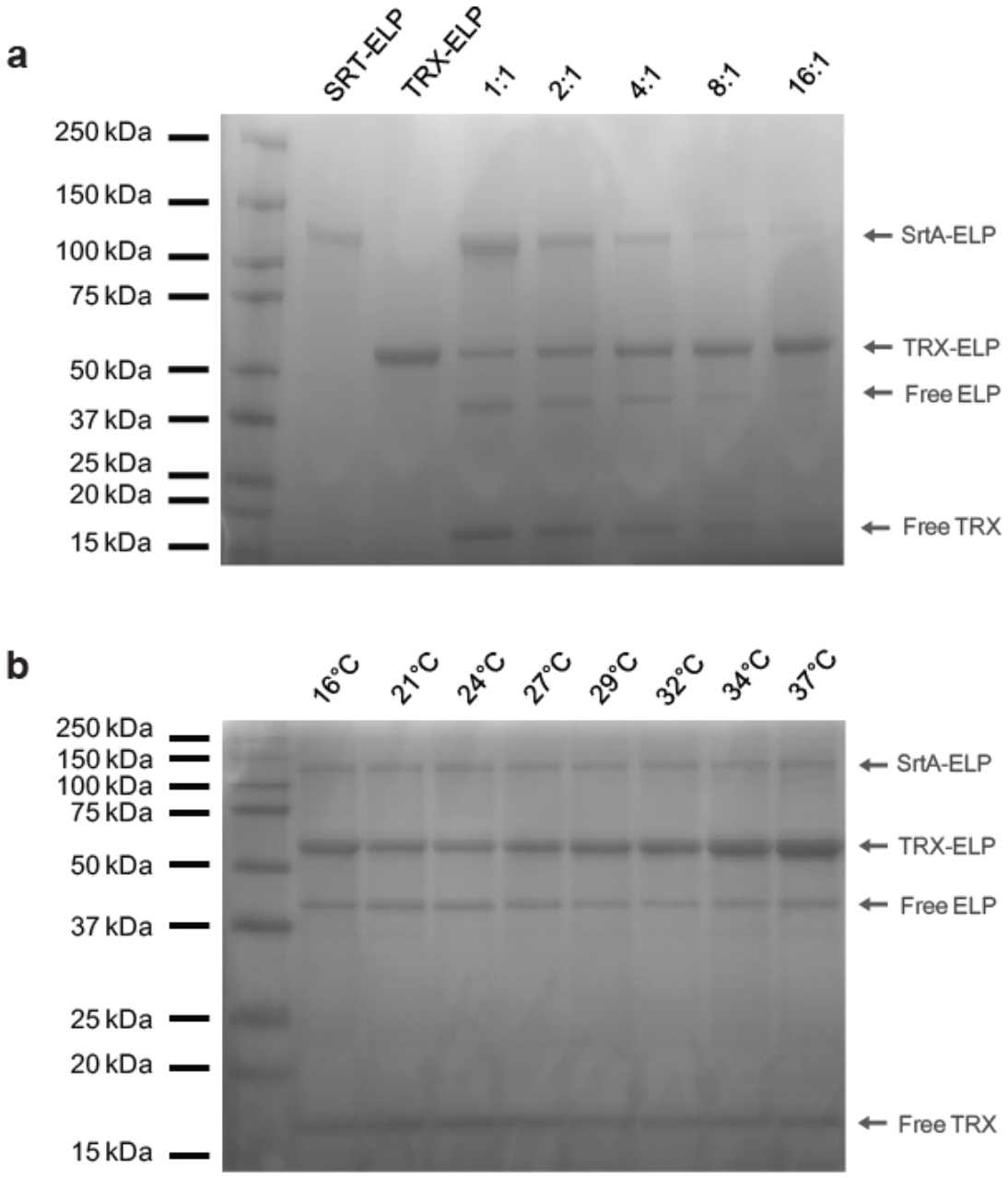

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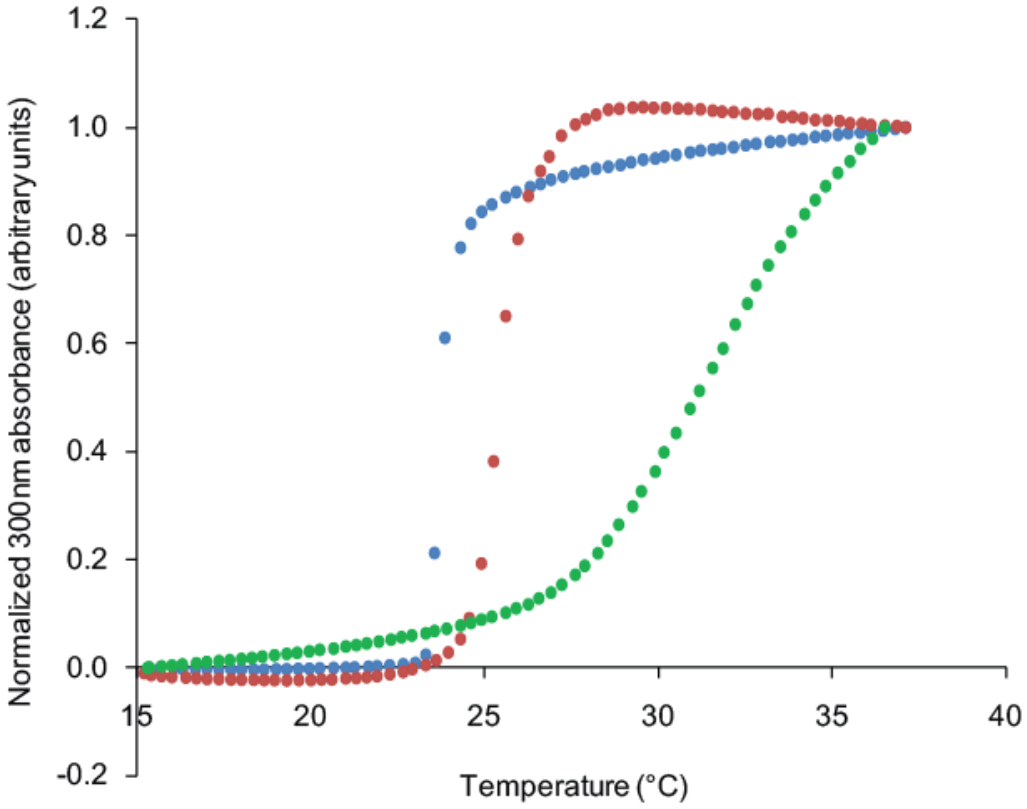

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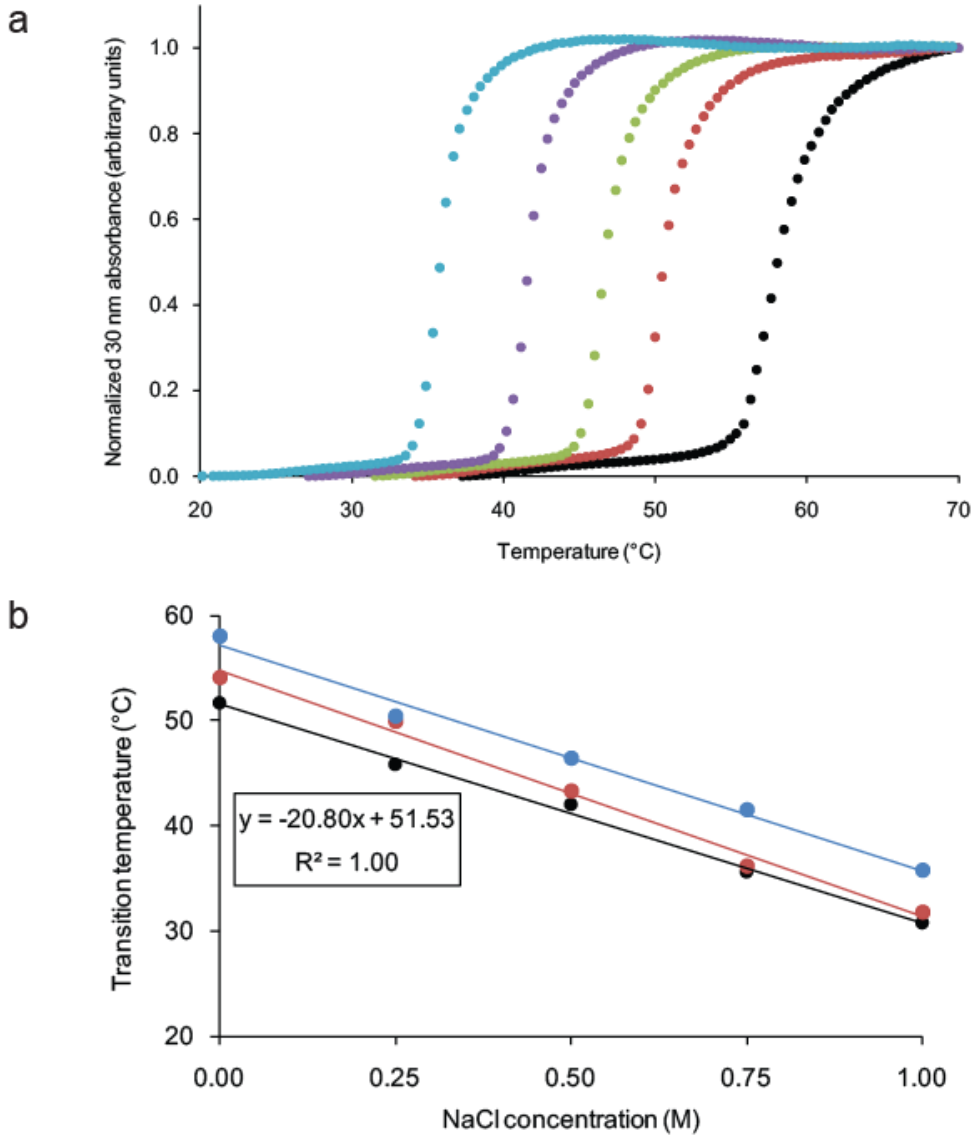

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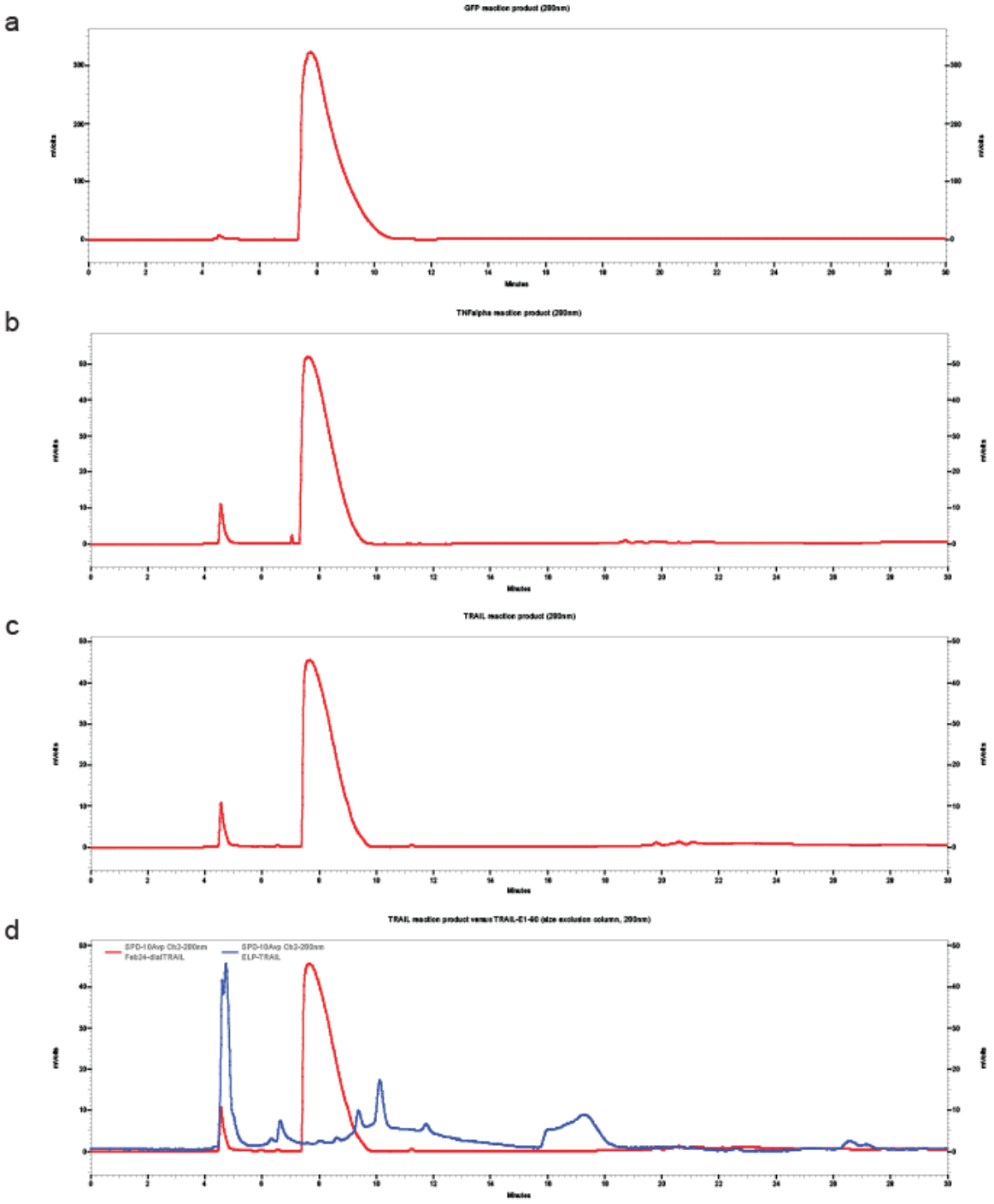

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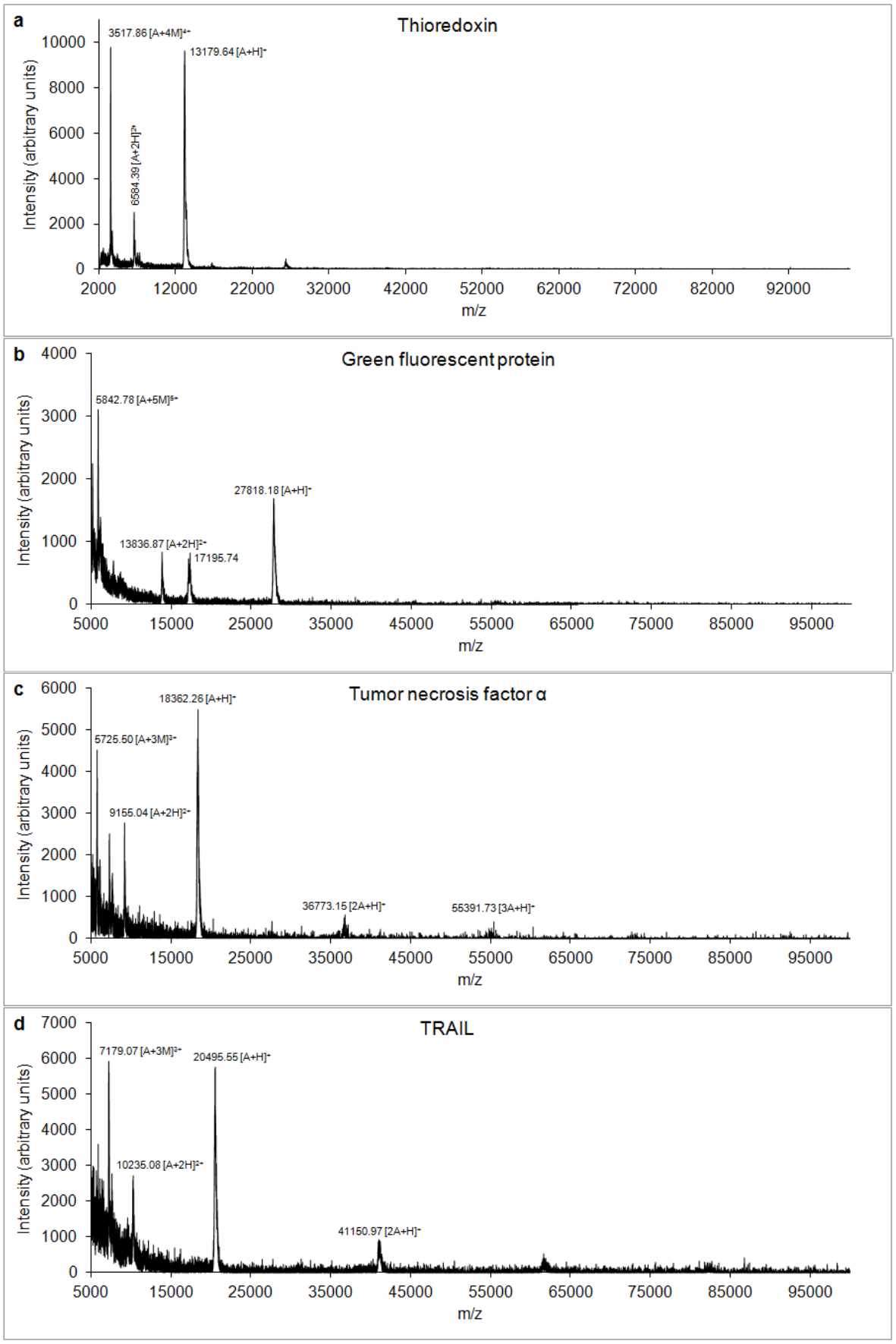

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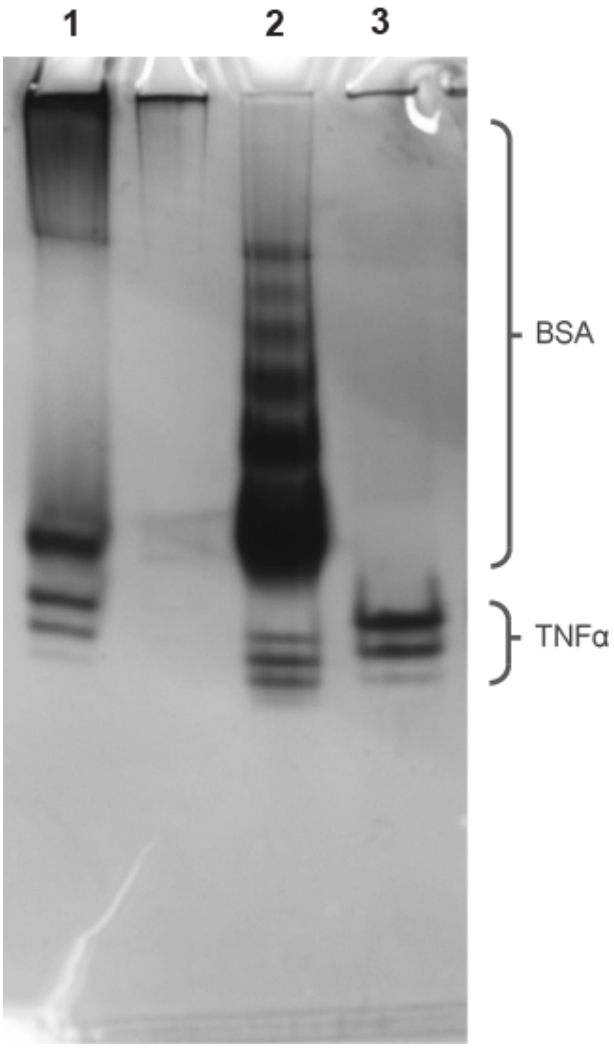

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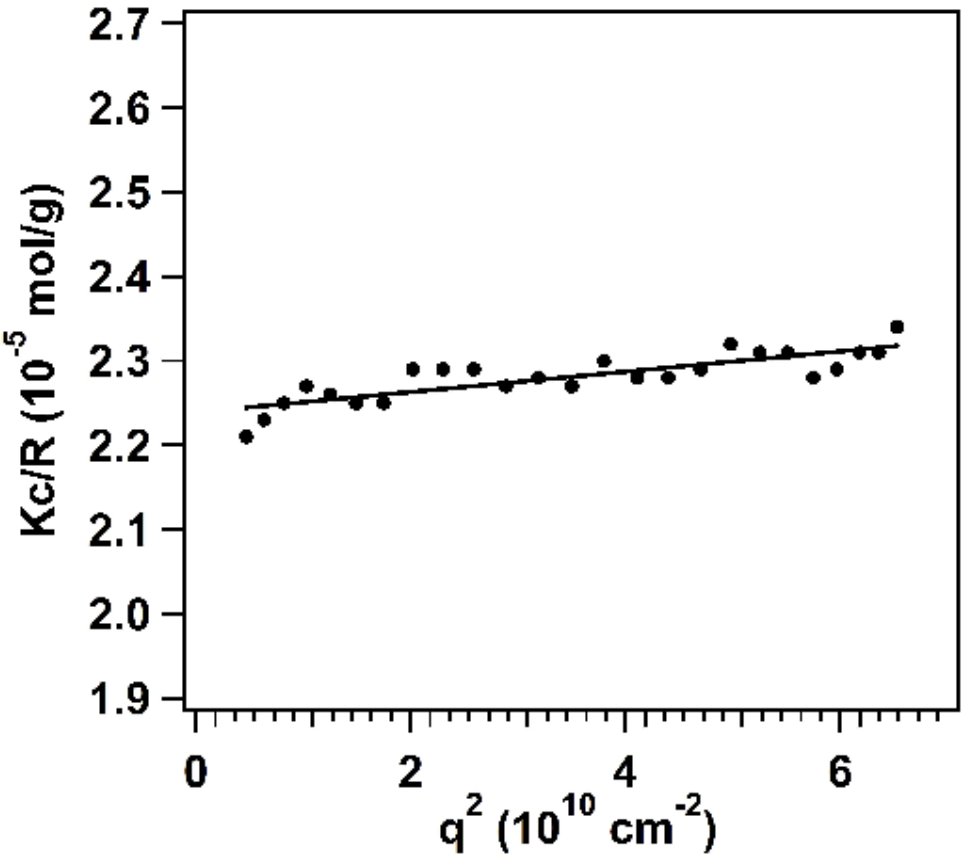

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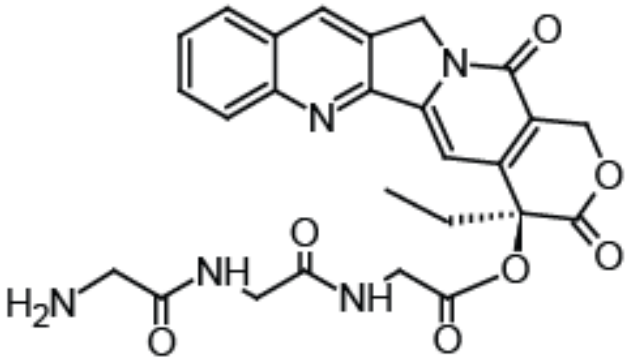

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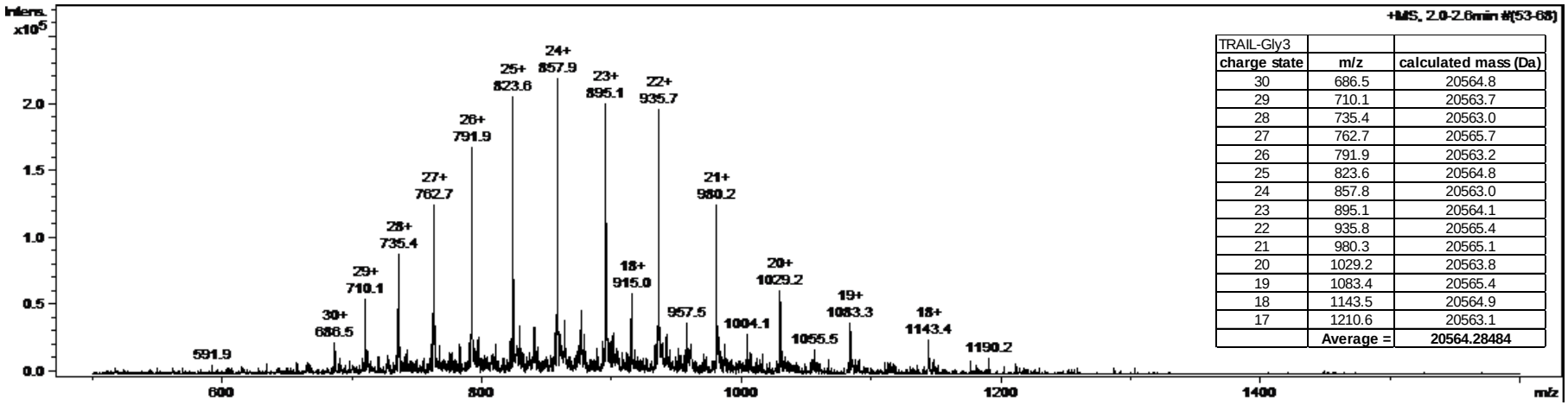
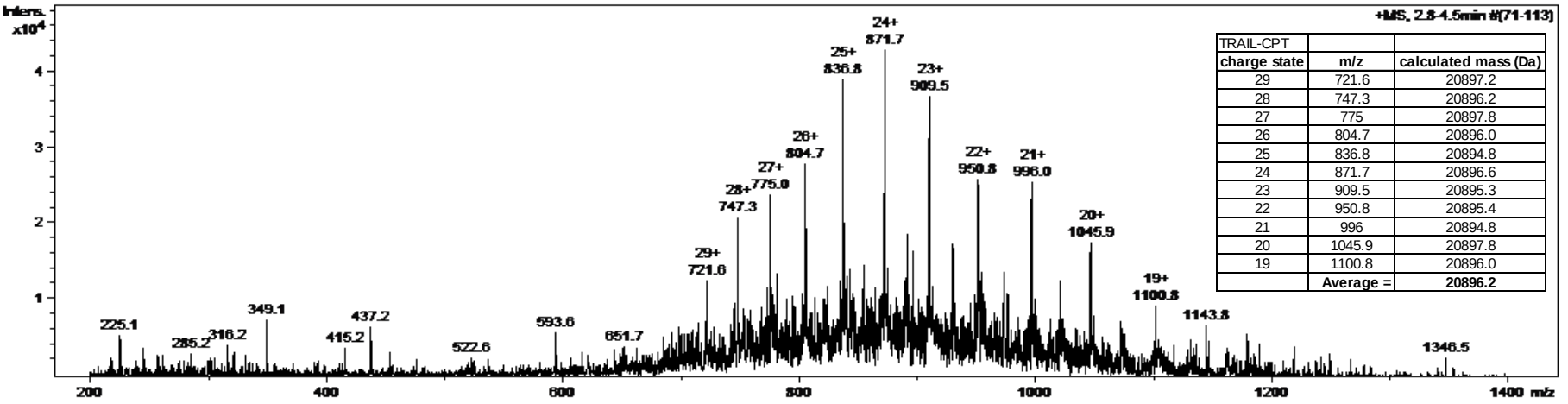
a



TRAIL-Gly3		
charge state	**m/z**	**calculated mass (Da)**
30	686.5	20564.8
29	710.1	20563.7
28	735.4	20563.0
27	762.7	20565.7
26	791.9	20563.2
25	823.6	20564.8
24	857.8	20563.0
23	895.1	20564.1
22	935.8	20565.4
21	980.3	20565.1
20	1029.2	20563.8
19	1083.4	20565.4
18	1143.5	20564.9
17	1210.6	20563.1
	Average =	**20564.28484**

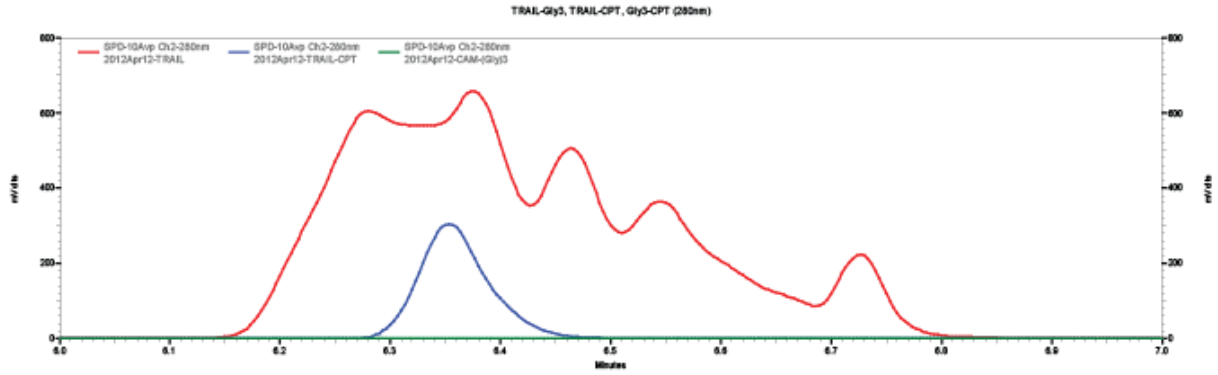
b



TRAIL-CPT		
charge state	**m/z**	**calculated mass (Da)**
29	721.6	20897.2
28	747.3	20896.2
27	775	20897.8
26	804.7	20896.0
25	836.8	20894.8
24	871.7	20896.6
23	909.5	20895.3
22	950.8	20895.4
21	996	20894.8
20	1045.9	20897.8
19	1100.8	20896.0
	Average =	**20896.2**

Supplementary Figure 11.

a



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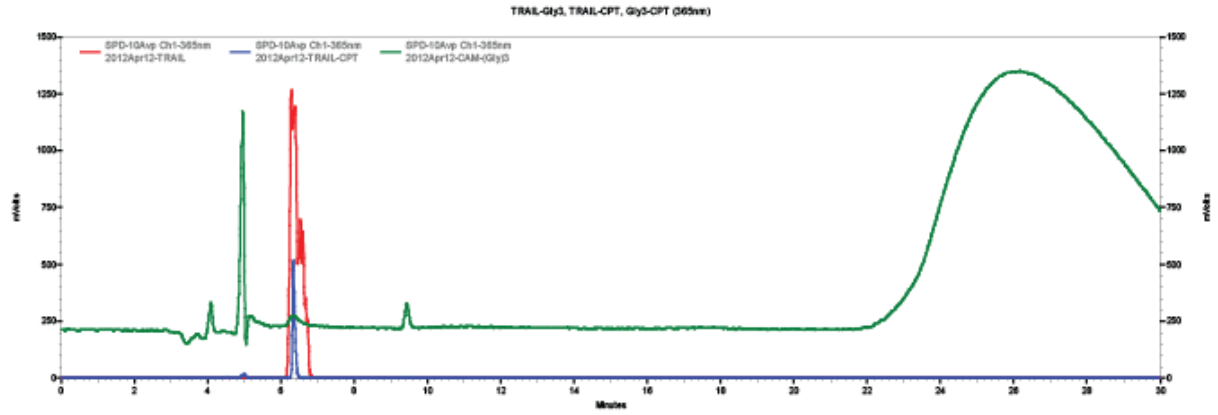

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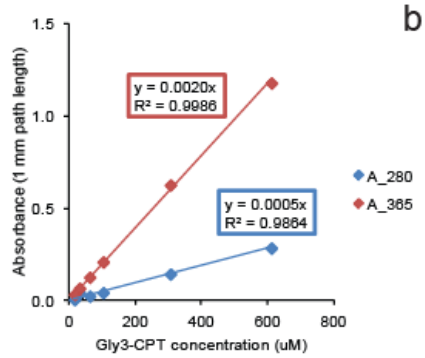
a

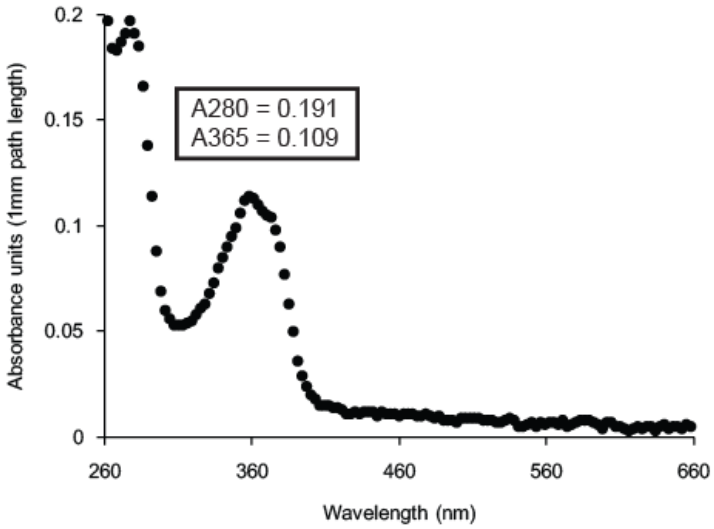


b

$$[CPT] = \frac{0.002\ uM}{A_{365\,nm}}$$

$$[TRAIL] = \frac{A_{280\,nm} - (0.0005\ uM^{-1})[CPT\ (uM)]}{\frac{1\ cm}{10\ mm}(1\ mm)(\varepsilon_{TRAIL}[M^{-1}cm^{-1}])(10^{-6}\frac{mol}{\mu mol})}$$

c



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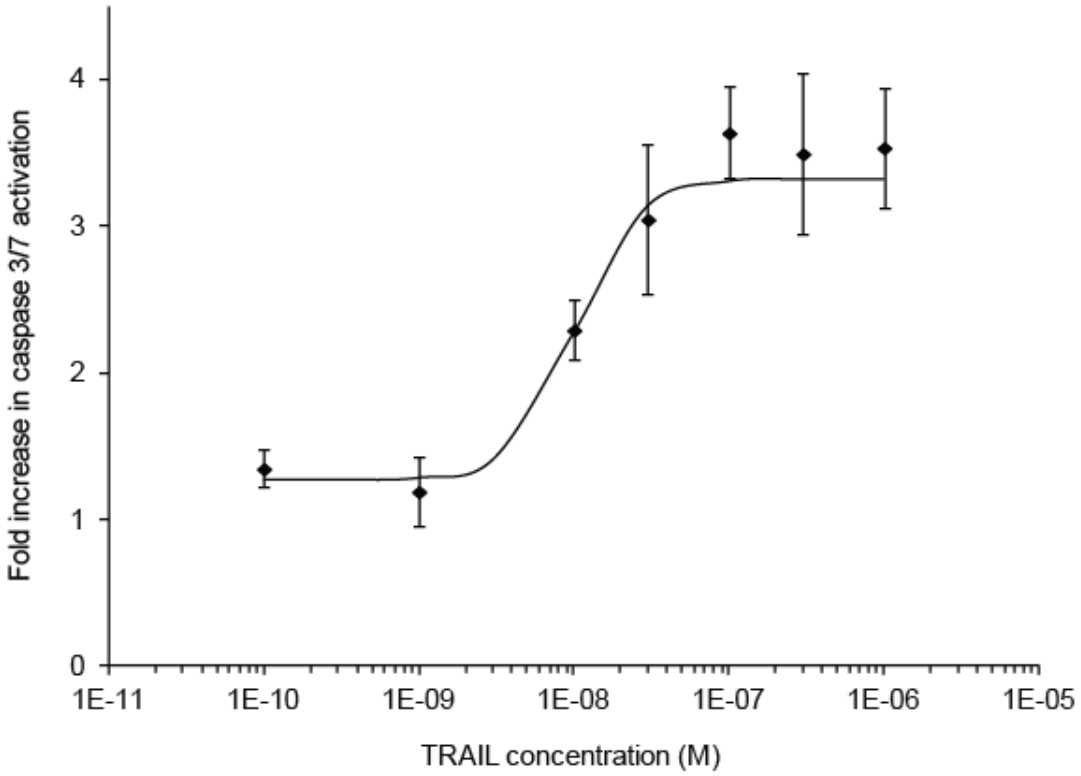

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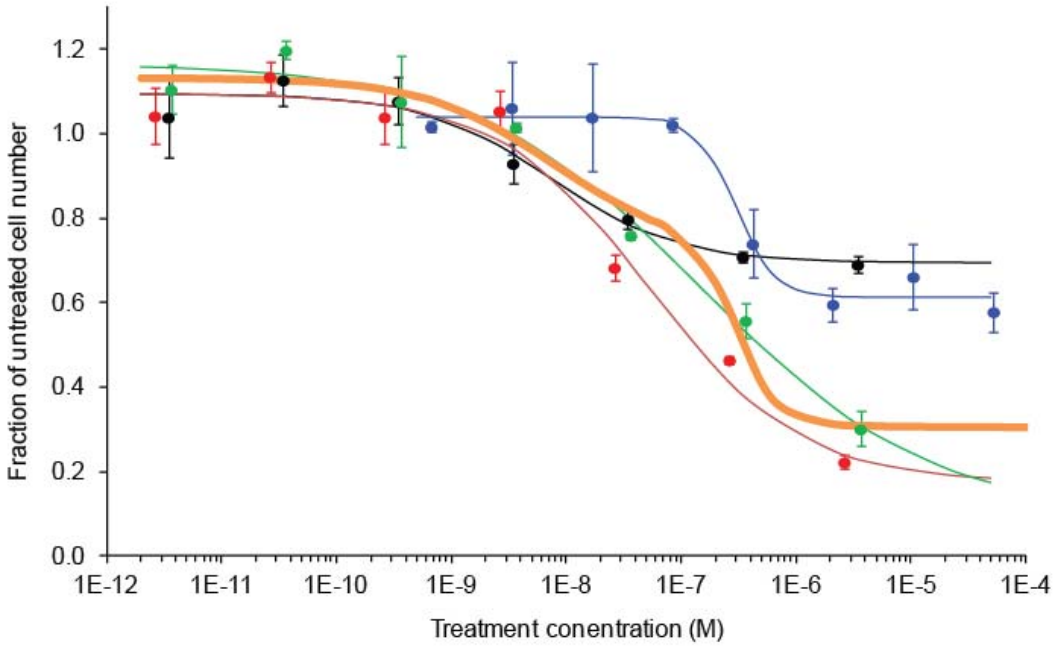

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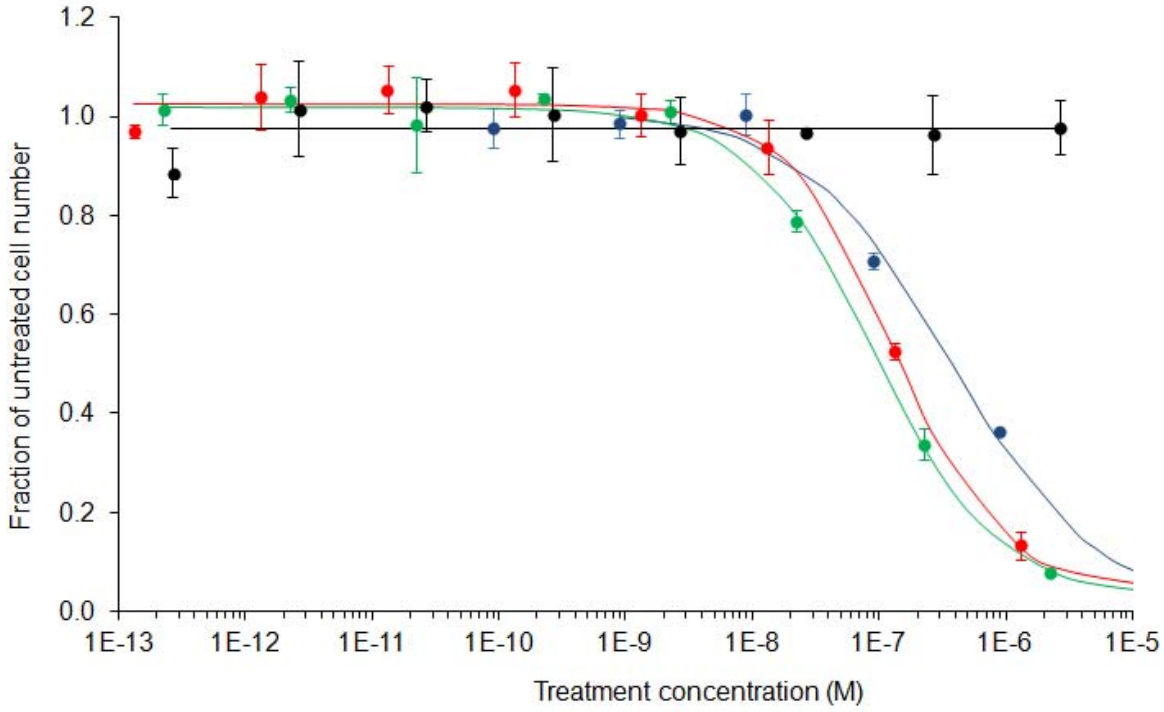

Supplementary Figure 16.

Detailed Experimental Methods

Fusion protein expression vectors

The full-length SrtA gene was cloned from *S. aureus* and the amino-terminal 59 amino acids were removed by polymerase chain reaction. DNA coding for the ELPs $(VPGVG)_{240}$, and $(VPGXG)_{90}$, where X represents A, G, and V in a molar ratio of 2:3:5 were constructed previously in our lab.

The genes for thioredoxin (TRX) and green fluorescent protein (GFP) were available from previous studies. DNA coding for soluble murine tumor necrosis factor α (TNFα, amino acids 80-235) and soluble human tumor necrosis factor-related apoptosis-inducing ligand (TRAIL, amino acids 114-281) were designed for *E. coli* codon usage and purchased from Life Technologies (Carlsbad, CA).

For SrtA-ELP, pET24 (Merck KGaA, Darmstadt, Germany) was modified with synthetic oligonucleotides from Integrated DNA Technologies (Coralville, IA) to allow in-frame insertion of SrtA at the 5' end of ELP $(VPGVG)_{240}$ with a short intervening linker. Target protein-ELP fusions were generated in a modified pET25 vector by in-frame addition of target proteins to the 5' end of ELP $(VPGXG)_{90}$ $[X=A_2G_3V_5]$ with a linker coding for the LPETG enzyme recognition site.

For ternary fusion constructs, the SrtA-ELP was transferred to pET25 (Merck KGaA). Target proteins were inserted in-frame at the 3' end of ELP $(VPGVG)_{240}$ with a linker containing the LPGAG enzyme recognition site.

Fusion protein expression and purification

Expression vectors were transformed into *E. coli* strain BL21 (DE3), purchased as chemically-competent cells from EdgeBio (Gaithersburg, MD). Clones were screened by colony PCR, sequenced, and subsequently maintained in 7% DMSO at -80°C. These stocks were used to inoculate a starter culture that was grown overnight at 37°C with orbital shaking at 250 rpm. All cultures were grown in terrific broth containing 45 μg/ml kanamycin or 100 μg/ml ampicillin for pET24 and pET25 constructs, respectively. The starters were centrifuged, resuspended in fresh media, and 2% inoculums were added to 4L unbaffled Erlenmeyer shake flasks containing 1L media with antibiotic. Cultures were grown for 6-8 hours with 200 rpm orbital shaking at 25°C and protein expression was induced by the addition of isopropyl β-D-1-thiogalactopyranoside (IPTG) to 0.5 mM final concentration. The culture temperature setpoint was changed to 16°C and induction was allowed to proceed overnight.

Cultures were centrifuged and resuspended in water at 1% of the original culture volume. Milli-Q water (EMD Millipore, Billerica, Massachusetts) was used throughout the purification of target protein-ELPs and SrtA-ELP. 5 mM Tris-Cl, 5 mM ethylene glycol tetraacetic acid (EGTA), pH 7.5 was used throughout ternary fusion purifications. Harvested cells were disrupted by sonication on ice using a Misonix Sonicator 3000 (Misonix, Inc., Farmingdale, NY). Polyethylenimine 10% (v/v) was added to the cell lysate to approximately 1% (v/v). Precipitated nucleic acids and insoluble host cell proteins were removed by centrifugation at 4°C. The supernatant was removed and the ELP phase transition was triggered by addition of ammonium sulfate to 0.2-0.3M. ELP fusion protein was pelleted by centrifugation, then resuspended in water or buffer. Centrifugation at 4°C removed any remaining insoluble material and retained the soluble ELP-fusion in the supernatant. The cycle of centrifugation with salt followed by cold centrifugation of the resuspended pellet was repeated 4-5 times to achieve desired protein purity as assessed by SDS-PAGE. Phase transitions for protein-ELP fusions were characterized using a Cary 300 Bio UV-Vis spectrophotometer (Agilent Technologies, Santa Clara, CA).

Sortase reactions

SrtA-ELP and target protein-ELP were combined in an enymze:substrate molar ratio of approximately 1:4. Protein concentrations were determined by the Beer-Lambert Law using calculated extinction coefficients and the absorbance at 280 nm measured using a Nanodrop ND-1000 spectrophotometer (Thermo Scientific, Wilmington, DE). Concentrated reaction buffer was added to a final concentration of 50 mM Tris-HCl, 150 mM NaCl, 10 mM $CaCl_2$, pH 7.5. Synthetic triglycine peptide from Alfa Aesar (Ward Hill, MA) was added to 10-20 molar excess over the target protein-ELP fusion and the reaction was allowed to proceed for approximately 18 hours at 20°C. Products were analyzed by SDS-PAGE.

For ternary fusion reactions, concentrated reaction buffer was added to 1x concentration and triglycine was added to 10-20 molar excess over the ternary fusion protein. The reaction was allowed to proceed overnight at 20°C.

Cleaved target proteins in both reaction designs were purified by centrifugation at 16.1 rcf in a fixed-angle benchtop centrifuge with temperature controlled at 40°C for 15 minutes. Prior to centrifugation, 1M sodium chloride was added to reactions where the target protein was originally fused to ELP $(VPGXG)_{90}$ $[X=V_5A_2G_3]$ to ensure the transition temperatures of the free ELP and unreacted target protein-ELP fusions were lowered to below 40°C.

Labeling reactions were identical to the reaction protocol for target protein purification using SrtA-ELP, except that triglycine was replaced with camptothecin modified by the Duke University Small Molecule Synthesis Facility (Durham, NC) to contain triglycine covalently linked to the E-ring hydroxyl group.

Protein characterization

SDS-PAGE was performed using 4-20% and 8-16% Tris-HCl gels purchased from Bio-Rad Laboratories (Hercules, CA) with 25 mM Tris, 192 mM glycine, 0.1% SDS running buffer and Laemmli sample buffer (Bio-Rad). Native PAGE was run using 4-20% Tris-glycine (TGX) gels from Bio-Rad with 25 mM Tris, 192 mM glycine running buffer and native sample buffer (Bio-Rad). Gels were stained with copper chloride and/or coomassie blue according to standard protocols.

Purified TRX, GFP, TNFα, and TRAIL were analyzed by HPLC (Shimadzu North America, Columbia, Maryland) using a Shodex KB-803 column (Showa Denko America, New York, New York) with a Milli-Q water mobile phase and isocratic

elution. TRAIL, TRAIL-CPT, and Gly_3-CPT were analyzed using an Aeris Widepore 3.6 μm XB-C18 column with a 70/30 (v/v) Milli-Q water/acetonitrile mobile phase and isocratic elution. Proteins were detected by absorbance at 280 nm and 365 nm using a SPD-10A UV-Vis detector (Shimadzu).

MALDI-TOFMS was performed using an Applied Biosystems Voyager-DE Pro system fitted with a nitrogen laser. All results were obtained using a sinapinic acid matrix in a 10:3 (v/v) ratio with the analyte.

ESI-MS was performed using an Agilent LC/MSD trap. Samples were prepared at 0.1 μM using Milli-Q water containing 0.3% formic acid and directly injected by infusion pump.

Static light scattering was performed using an ALV CGS-3 goniometer (ALV, Langen, Germany). Samples were filtered through 20 nm filters with no concentration loss as measured by absorption at 280 nm. All testing was conducted at room temperature. Static measurements were made in triplicate for 15 seconds each from 30° to 150° in 5° increments.

TNFα and TRAIL cytotoxicity assays

L929 cells were obtained from the Duke University Health System Cell Culture Facility (Durham, North Carolina) and maintained in RPMI 1640 supplemented with 10% fetal bovine serum, 1 mM sodium pyruvate, 5 mM HEPES, and 100 units penicillin/100 μg streptomycin per mL (all from Life Technologies, Grand Island, New York). Cells were inoculated in 96-well flat-bottom culture dishes at a density of 50,000 cells/mL and treated with a range of concentrations of TNFα purified by cleavage of TNFα-ELP, from the ternary fusion, or purchased from eBioscience (San Diego, CA). Negative control groups were included that were untreated. Cultures were grown at 37°C under 5% CO_2 for 36 hours.

MDA-MB-231 cells were obtained from the Duke University Health System Cell Culture Facility and maintained in DMEM:F12 (1:1) supplemented with 10% fetal bovine serum and 100 units penicillin/100 μg streptomycin per mL (Life Technologies). Cells were inoculated into 96-well flat-bottom culture dishes at a density of 100,000 cells/mL and incubated for approximately 18 hours. The growth media was aspirated and replenished with serum-free DMEM:F12 (1:1) and TRAIL, Gly_3-CPT, TRAIL-CPT conjugate, or non-conjugated CPT and TRAIL (0.8:1 mol/mol) was added at a range of concentrations. Cell viability relative to an untreated control group was assessed after 24 hour incubation with treatment.

Growth inhibition versus the untreated controls was assessed using a CellTiter 96 One Solution MTS/PMS viability assay purchased from Promega (Madison, WI) and used per the manufacturer's instructions. All treatment concentrations were repeated in triplicate, and the means for each group were normalized to that of the untreated group. All samples were further corrected for background absorbance by measuring the absorbance of the MTS/PMS reagent added to cell-free media. Dose-response data was fit to a 4-parameter logistic model using Wolfram Mathematica version 8.0 (Wolfram Research, Inc., Champaign, IL).

For caspase activation assays, MDA-MB-231 cells were seeded in 96-well flat-bottom culture plates at 100,000 cells/mL and allowed to grow for 18 hours. The media was changed and TRAIL purified from cleavage of TRAIL-ELP was added at a range of concentrations. Caspase 3 and 7 activation was quantified after 5 hours of treatment using the Caspase-Glo 3/7 assay (Promega) per the manufacturer's instructions. All treatment groups were normalized to a media-only control and caspase activation in TRAIL-treated groups was reported relative to the untreated control.