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Protein Expression

Three-in-One Chromatography-Free Purification, Tag Removal, and Site-Specific Modification of Recombinant Fusion Proteins Using Sortase A and Elastin-like Polypeptides**

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Column chromatography is the workhorse of protein purification, but the requirements for large volumes of buffers, expensive resins that have limited potential for re-use, and significant operator hours to optimize and execute the separations present major financial and technical hurdles to scaling up production. Additionally, covalent modification of proteins with small molecules and polymers is routine, but available conjugation methods frequently produce heterogeneous products as a result of incomplete reactivity or the presence of multiple reaction sites within a protein. These two unit operations currently present major limitations to the production of recombinant proteins at the scales necessary for both research and manufacturing.

A simple process that would facilitate efficient purification as well as site-specific, covalent conjugation of small molecules to a target protein would be highly useful in biopharmaceutical production. Herein, we present a "three-in-one" method that uses the transpeptidase activity of *Staphylococcus aureus* Sortase A (SrtA) in tandem with elastin-like polypeptides (ELPs) to enable: 1) recombinant fusion-protein purification without chromatography, 2) removal of the ELP fusion tag and facile, chromatography-free recovery of pure target protein, and 3) site-specific covalent coupling of an extrinsic moiety to the purified target protein concurrent with cleavage from its ELP fusion partner. We present two complementary fusion protein designs that achieve these goals. The entire process provides a general method for the purification and modification of a variety of recombinant proteins that is simple, robust, and scalable.

Expressing a protein as a fusion with another protein or a peptide tag is a widely-used strategy for purification by affinity chromatography.[1–4] Elastin-like polypeptides (ELPs) are purification tags that have been developed by our research group to provide convenient fusion-protein purification without requiring chromatography.[5,6] ELPs are peptide polymers composed of a repeated VPGXG pentapeptide unit, where X is any amino acid except proline. They can be designed to reversibly aggregate above a specific solution temperature—their inverse transition temperature (T_t)—by specifying the ELP amino acid composition, the ELP chain length, and the type and concentration of salt in solution.[7] This reversible phase-transition behavior is retained by ELP fusion proteins and forms the basis for their purification by centrifugation rather than chromatography (Scheme 1a).[8,9]

Although purification is initially simplified by fusion of the protein to a tag, in many applications the tag must be removed because it affects the bioactivity of the target protein. This is accomplished by including a protease site or a self-cleaving intein between the target protein and the tag.[10,11] However, the ease of purification provided by the tag is lost upon cleavage of the fusion, and isolation of the target protein from the digested product requires additional chromatographic separation steps that must be customized for a given target.

We hypothesized that a SrtA-ELP fusion protein could be used to cleave other ELP fusion proteins, and that the released target proteins could be easily recovered at high purity by another round of phase-transition-mediated purification, without chromatography (Scheme 1b). We constructed a gene-level fusion of SrtA lacking its 59 amino-terminal residues—a truncation that has been shown to have no impact on its transpeptidase activity[12]—to an ELP with the sequence $(VPGVG)_{240}$. A panel of target proteins including thioredoxin (TRX), green fluorescent protein (GFP), 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 fused at the gene level to a different ELP, $(VPGXG)_{90}$, where X represents alanine (A), glycine (G), and valine (V) in a molar ratio of 2:3:5. These target proteins range from well-behaved, single sub-unit proteins (TRX and GFP) to more difficult to express, pharmaceutically relevant proteins that form stable quaternary structures (TNFα and TRAIL). The linker between all the target proteins and their ELP fusion partners contained the LPETG motif (Supporting Information, Figure S1), which was previously demonstrated to be the optimal recognition sequence for SrtA.[13]

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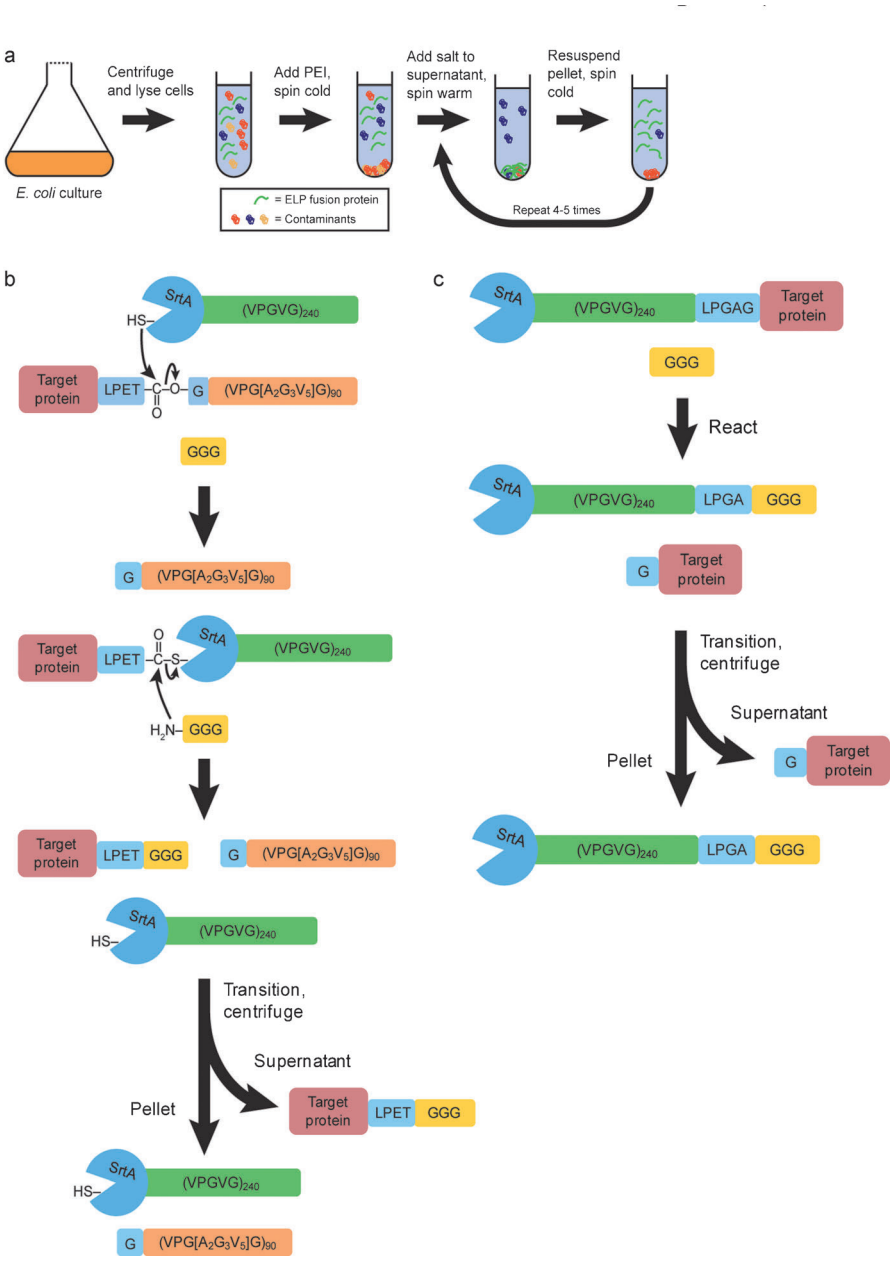

Scheme 1. a) Overview of ELP fusion-protein purification by inverse transition cycling. Polyethyleneimine (PEI) was used to precipitate the bacterial DNA. b) Scheme of the reaction catalyzed by SrtA-ELP for the recovery and labeling of target proteins from binary ELP fusions. c) Strategy for target recovery and labeling by reaction of ternary SrtA-ELP-target-protein fusions.

We also developed a ternary fusion in which SrtA and the target protein were linked by the ELP $(VPGVG)_{240}$. This design allowed for straightforward purification of the target protein without the need to add extraneous SrtA-ELP (Scheme 1c). Use of the optimal LPETG enzyme recognition site produced no intact ternary fusion because of premature cleavage during expression. However, amino acid substitutions in the LPXTG motif have been shown to lower the SrtA reaction rate,[13] and we identified a variant—LPGAG—that allowed expression of the intact ternary fusion protein when the protein was expressed at low temperature and the purification buffer contained ethylene glycol tetraacetic acid (EGTA) to scavenge the Ca^{2+} ions required for SrtA activity[12] (Figure S2).

All fusion proteins were expressed in *Escherichia coli* (*E. coli*) and were purified by inverse transition cycling (ITC), a method that we have previously developed.[6,8,9] We designed an ITC method that consists of centrifugation of the cell lysate at 4 °C in low-salt buffer where the ELP fusion is below its T_t and soluble, collection of the supernatant, centrifugation at 30 °C in 0.3 M ammonium sulfate where the ELP fusion is above its T_t and insoluble, followed by collection and solubilization of the pellet—containing the fusion protein—in cold, low-salt buffer. Repeated cycles of cold and warm centrifugation led to enrichment of the ELP fusion protein by eliminating other *E. coli* proteins that do not exhibit reversible phase-transition behavior.

Target protein-ELP fusions and SrtA-ELP were expressed and purified separately, then co-incubated in a buffer that contained Ca^{2+} ions and triglycine. For ternary SrtA-ELP-target protein fusions, the cleavage reaction was initiated by transfer of the fusion protein into a buffer that contained Ca^{2+} ions and triglycine in the last stage of the ITC purification. In both systems, the target protein was released by cleavage of the fusion at the SrtA recognition site. Based on initial studies (Figure S3), we selected conditions of overnight incubation at 20 °C and a 1:4 enzyme/target molar ratio for cleavage of target-protein-ELP fusions by SrtA-ELP.

To isolate the target protein, the phase transitions of all other reaction products—SrtA-ELP, uncleaved target protein-ELP fusions, and free ELP—were triggered by adding sodium chloride to 1 M and heating to 40 °C. These conditions were selected to ensure aggregation of the SrtA-ELP and unreacted TRX-ELP—one of our more hydrophilic fusion proteins—so that a common method could be used for all of the target proteins in our study (Figures S4, S5).

Dialysis or diafiltration of the supernatant removed the remaining triglycine and left behind the purified target protein. Figure 1a shows sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) of the unpurified reaction product and centrifugation fractions for the purification of TRX, GFP, TNFα, and TRAIL. Figure 1b shows similar analysis for GFP and TNFα purified from ternary

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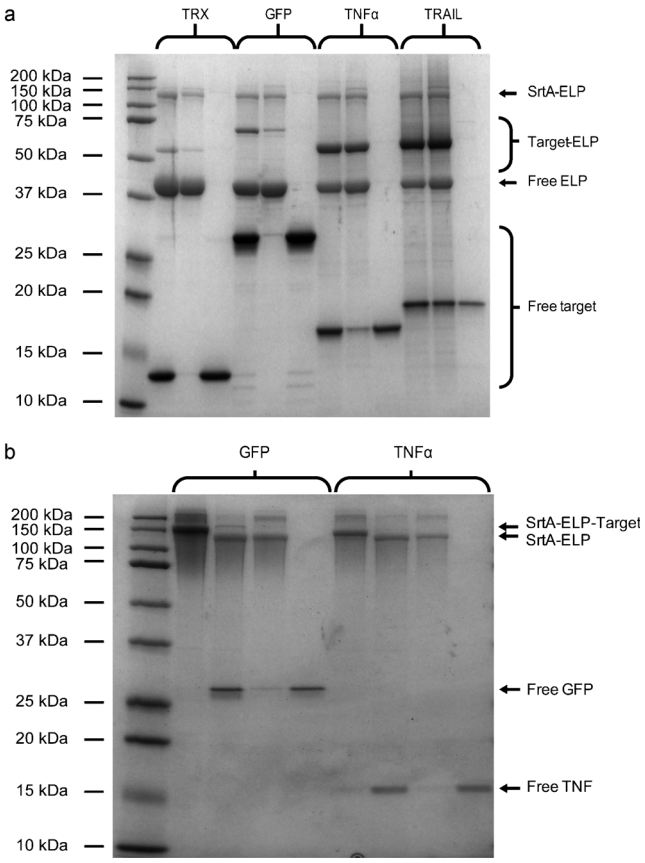

Figure 1. a) SDS-PAGE of target protein purification by reaction of target-protein-ELP and SrtA-ELP fusions. Lanes from left to right, for each target protein: reaction product, centrifugation pellet, and centrifugation supernatant. b) SDS-PAGE of ternary fusion reactions. Lanes from left to right for each target protein: unreacted ternary fusion, reaction product, centrifugation pellet, and centrifugation supernatant.

fusion reactions. We observed excellent cleavage and target-protein recovery (Table 1). High-performance liquid chromatography (HPLC) confirmed that the purities of the target proteins were greater than 95% (Figure S6) and MALDI-TOF mass spectrometry confirmed the molecular weight of each target protein (Figure S7).

The fluorescence emission spectrum of GFP provided a simple indication that our method gave properly folded, active protein. TNFα was investigated as a more complicated case, as homotrimer assembly is critical for TNFα signaling.[14,15] Non-denaturing, native PAGE was used to evaluate the quaternary structure of the protein versus commercially available TNFα. Protein from both sources ran similarly and appeared as three distinct bands on the gel, suggestive of monomers, dimers, and trimers (Figure 2b). The commercial preparation contains 0.5% bovine serum albumin, which accounts for the lower-mobility bands at the top of the gel (Figure S8). Interestingly, MALDI-TOF MS not only confirmed the mass of the monomeric TNFα, but also showed peaks at double and triple the monomer m/z, which provided additional evidence of the proper quaternary structure (Figure S7). Static light scattering led to an average molecular weight of 44.7 kDa for TNFα purified from the ternary fusion protein (Figure S9), which is consistent with the average molecular weight of 49 kDa reported previously for TNFα.[16]

Table 1: Summary of reactions of target protein-ELPs and SrtA-ELP for representative batches of each fusion protein.[a]

Target protein	SrtA reaction efficiency [%]	Target protein recovery [%]	Target protein purity[b] [%]	Target protein yield [mg L^{-1} of fermentation]
TRX	88	100	98	35
GFP	85	100	100	28
TNFα	54	82	96	16
TRAIL	46	46	95	9

[a] Conversion and recovery percentages were calculated by analysis of SDS-PAGE images and confirmed by quantifying the purified reaction product concentrations by spectrophotometry. [b] Determined by HPLC.

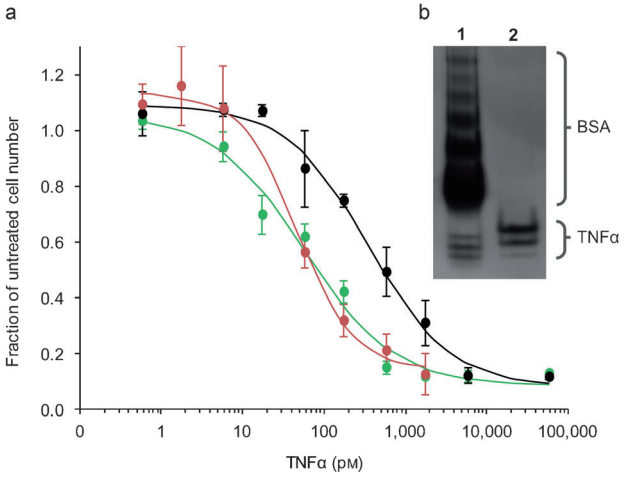

Figure 2. a) Viability of L929 cells after incubation with TNFα, from reaction of TNFα isolated from the binary fusion (red), from the ternary fusion (black), or purchased from eBioscience (green) relative to an untreated control. Error bars = standard deviations. b) Native PAGE of commercial TNFα (lane 1) and TNFα from the ternary fusion (lane 2).

We examined the bioactivity of the recombinant TNFα by measuring its effect on the L929 mouse fibrosarcoma cell line, which is known to be sensitive to cytolysis by TNFα at picomolar concentrations.[17,18] The number of cells present relative to an untreated group was assessed for cells treated with SrtA-purified or commercial TNFα (Figure 2a). ED_{50} values were 48 pM, 350 pM, and 57 pM, for TNFα isolated from cleavage of TNFα-ELP by SrtA-ELP, TNFα isolated from the ternary fusion, and commercial protein, respectively. The similar bioactivity of the commercial and SrtA-purified TNFα further reinforced that there was proper folding and multimerization of the protein, as suggested by native PAGE, static light scattering, and MALDI-TOF MS.

SrtA, unlike proteases or self-splicing inteins that simply cleave a target protein from a tag, also enables site-specific, covalent attachment of other molecules to the target protein. Our protein-purification reactions were designed to mimic a typical protease digestion by using synthetic triglycine as

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a nucleophile. However, triglycine-modified lipids and small molecules, as well as proteins with amino-terminal glycine residues have also been used effectively as nucleophiles in transpeptidation reactions catalyzed by Sortase A.[19–23] In principle, our system is flexible in that it can be easily extended to accommodate these nucleophiles. Moreover, this conjugation approach has the attractive feature that only one molecule is installed specifically at the C-terminus of the protein.

To demonstrate the flexibiity of our system, we conjugated the chemotherapeutic drug camptothecin (CPT) to TRAIL, generating a hybrid anticancer agent. We chose CPT for its potent antitumor activity and because it is a chromophore with an extinction peak at 365 nm, which allowed the product to be tracked spectrophotometrically. CPT was modified such that the hydroxy group on the E-ring was coupled to the carboxy terminus of triglycine (Figure S10).

The conditions used for the TRAIL-CPT conjugation reaction and product recovery were the same as those used for the purification of unlabeled protein, except that Gly_3-CPT was substituted for triglycine. SDS-PAGE of parallel reactions with triglycine or Gly_3-CPT (Figure 3a) indicated significant conversion in both cases, suggesting that Gly_3-CPT was an effective nucleophile. Based on analysis of the SDS-PAGE images, we determined that the reaction conversion using Gly_3-CPT was approximately 80% of that for a reaction using Gly_3. Similarly, the overall recovery after reaction purification for the Gly_3-CPT reactions were consistently 65% of the recoveries for reactions using Gly_3. We attribute the lower yield and recovery to the lower solubility of the CPT moiety, which likely lowered its concentration in the reaction and possibly contributed to some nonspecific aggregation of the product.

ESI-MS confirmed that the m/z ratios of TRAIL and the TRAIL-CPT conjugate differed by the mass of a single molecule of CPT (Table 2 and Figure S11). HPLC in 70:30 (v/v) Milli-Q water/acetonitrile indicated that the conjugated and non-conjugated compounds eluted differently, and that excess triglycine-CPT was successfully removed by diafiltration of the recovered reaction product (Figure S12).

Notably, in our reaction design the unreacted protein retained the ELP tag, so that a homogeneous conjugate could be purified from unreacted protein by a single centrifugation step, regardless of the extent of reaction conversion. Based on a calibration curve for Gly_3-CPT (Figure S13), we consistently obtained conjugation ratios greater than 0.8:1 (moles of CPT/moles of TRAIL). The controlled stoichiometry and homogeneous product produced by our conjugation method, as well as the ability to easily separate the conjugate from unreacted protein are especially important for pharmaceutical applications, where non-specific or incomplete reactivity must be controlled precisely to achieve batch-to-batch consistency for regulatory approval and patient safety.

Bioactivity of the TRAIL-CPT conjugate was assessed by measuring apoptosis in the TRAIL-sensitive human breast adenocarcinoma cell line MDA-MB-231 (Figure 3b). The number of viable cells relative to an untreated control (MTS assay) was assessed after incubation with TRAIL, Gly_3-CPT, TRAIL-CPT conjugate, or TRAIL and Gly_3-CPT in a molar ratio equivalent to that of the conjugate. High concentrations of Gly_3-CPT alone killed 40% of the cells, whereas TRAIL killed a maximum of 30% of the cells and activated Caspases 3 and 7 in a dose-dependent manner (Figure S14). Notably, the EC_{50} value determined from the caspase-activation assay confirmed the value determined by the MTS assay.

Dosing conjugated TRAIL-CPT decreased the number of viable cells by a maximum of 75%. Although improved cell

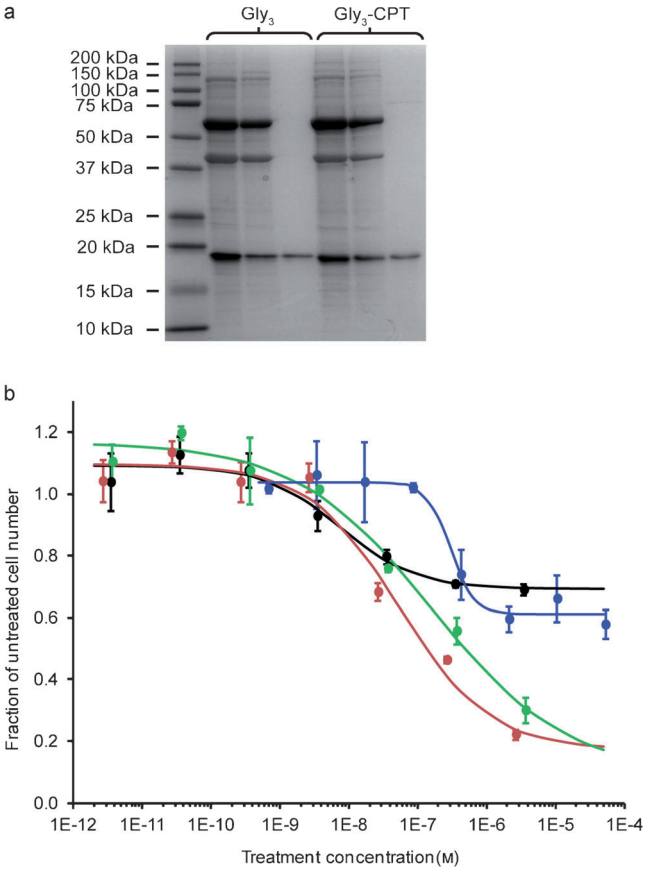

Figure 3. a) SDS-PAGE analysis of TRAIL-ELP reactions run in parallel with triglycine (Gly_3) or triglycine-modified camptothecin (Gly_3-CPT). Lanes from left to right, for each reaction: raw product, centrifugation pellet, and centrifugation supernatant. b) Cell death in MDA-MB-231 cells treated with TRAIL (black), Gly_3-CPT (blue), TRAIL-CPT conjugate (red), or a mixture of Gly_3-CPT and TRAIL at a 0.8:1 molar ratio (green). For combination treatments, the TRAIL concentration is indicated. The viable cell number is reported relative to an untreated group. Error bars = standard deviations.

Table 2: Summary of ESI mass spectrometry data for TRAIL and TRAIL-CPT purified from TRAIL-ELP by reaction with SrtA-ELP.[a]

Species	Predicted M_w [Da]	ESI-MS M_w [Da]
TRAIL-Gly_3-CPT	20893.2	20896.2
TRAIL-Gly_3	20563.9	20564.3
Difference (CPT)	329.3	331.9

[a] Molecular weights are reported as the average of 14 and 11 different charge states for TRAIL-Gly_3 and TRAIL-CPT, respectively.

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killing was anticipated using a combination of CPT and TRAIL, it is noteworthy that the TRAIL-CPT conjugate had the same potency and efficacy as the combination of non-conjugated Gly_3-CPT and TRAIL in an equivalent molar ratio, which suggested that each molecule within the conjugate retained its activity. Interestingly, both conjugated and non-conjugated drug combinations showed an additive effect (Figure S15). We also tested the bioactivity of the TRAIL-CPT conjugate on the TRAIL-insensitive human prostate adenocarcinoma cell line PC3 (Figure S16). Both TRAIL-CPT and a combination of Gly_3-CPT and TRAIL killed cells in a manner equivalent to Gly_3-CPT alone over the concentration range tested, suggesting that these cells did not become sensitized to TRAIL by co-treatment with CPT. Although the conjugation of TRAIL and CPT does not offer an advantage in our cytotoxicity assays, it nonetheless provides a proof-of-concept example of the ease with which bioactive combination drugs can be produced using our system.

The combination of SrtA and ELPs represents a powerful and flexible system for the purification and site-specific chemical modification of proteins. As with inteins, our ternary fusion provides a straightforward, all-in-one system that is subject to premature cleavage during expression. However, our binary fusion system provides an alternative with excellent control over reactivity, high product yields, and virtually no increase in complexity.

Using our reaction strategies, significant quantities of highly pure, bioactive recombinant proteins can be made without column chromatography using a method that is practical and applicable to a variety of target proteins. The ease with which our method can be executed, and the flexibility to perform an optional, one-step site-specific conjugation reaction that homogeneously labels the product, makes this a valuable, three-in-one technique for the production of therapeutic proteins and protein–small-molecule conjugates.

Experimental Section

Fusion protein genes: The SrtA gene was cloned from *S. aureus* minus the amino-terminal 59 amino acids by polymerase chain reaction. DNA sequences to encode 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 TRX and GFP were available from previous studies. DNA sequences for soluble murine TNFα (amino acids 80–235) and soluble human TRAIL (amino acids 114–281) were designed for *E. coli* codon usage and purchased from Life Technologies (Carlsbad, CA).

Fusion protein expression and purification: Expression vectors were transformed into the *E. coli* strain BL21 (DE3). Frozen stocks were used to inoculate a starter culture that was grown overnight at 37 °C with orbital shaking at 250 rpm. Starter cultures were centrifuged, resuspended in fresh media, and used to inoculate 4 L shaking flasks containing 1 L terrific broth with the appropriate antibiotic (2 % inoculums were used). All cultures were allowed to grow for 6–8 h with 200 rpm orbital shaking at 25 °C. Protein expression was induced by the addition of isopropyl β-D-1-thiogalactopyranoside (IPTG) to final concentration of 0.5 mM. Induction proceeded overnight at 16 °C. Cells were lysed by sonication and fusion proteins were recovered by inverse transition cycling.

Sortase reactions: SrtA-ELP and target protein-ELP were combined to achieve an enymze:substrate molar ratio of approximately 1:4. Protein concentrations were determined by the Beer–Lambert Law using calculated extinction coefficients[24] and the absorbance at 280 nm measured by a Nanodrop ND-1000 spectrophotometer (Thermo Scientific, Wilmington, DE). 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 was added to 10–20 molar excess over the target protein-ELP fusion and the reaction was allowed to proceed for approximately 18 h at 20 °C.

Cleaved target proteins in both reaction designs were purified by centrifugation at 16.1 RCF in a fixed-angle benchtop centrifuge at 40 °C for 15 min. Prior to centrifugation, 1M sodium chloride was added to reactions where the target protein was originally fused to the ELP $(VPGXG)_{90}$ $(X = V_5A_2G_3)$.

Labeling reactions were identical to the method 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 hydroxy group of camptothecin.

Cytotoxicity assays: L929 cells were cultured in 96-well culture dishes at an initial density of 50000 cells mL^{-1} 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 incubated for 36 h.

MDA-MB-231 cells were cultured in 96-well culture dishes at an initial density of 100000 cells mL^{-1} and incubated for approximately 18 h. The growth media was replaced with serum-free media and TRAIL, Gly_3-CPT, TRAIL-CPT conjugate, or non-conjugated CPT and TRAIL (0.8:1 mol/mol) were added.

Growth inhibition versus untreated controls was assessed using a CellTiter 96 One Solution MTS/PMS viability assay purchased from Promega (Madison, WI). Absorbance was measured at 490 nm in a multi-well spectrophotometer. All treatment concentrations were repeated in triplicate, and the mean average of each group was normalized to that of the untreated group. All samples were corrected for background absorbance by measuring the absorbance at 490 nm of the MTS/PMS reagent added to the cell-free media.

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[1] P. Braun, Y. Hu, B. Shen, A. Halleck, M. Koundinya, E. Harlow, J. LaBaer, *Proc. Natl. Acad. Sci. USA* **2002**, *99*, 2654 – 2659.
[2] Q. Hang, L. Woods, M. Feiss, C. E. Catalano, *J. Biol. Chem.* **1999**, *274*, 15305 – 15314.
[3] E. de Boer, P. Rodriguez, E. Bonte, J. Krijgsveld, E. Katsantoni, A. Heck, F. Grosveld, J. Strouboulis, *Proc. Natl. Acad. Sci. USA* **2003**, *100*, 7480 – 7485.
[4] G. Rigaut, A. Shevchenko, B. Rutz, M. Wilm, M. Mann, B. Séraphin, *Nat. Biotechnol.* **1999**, *17*, 1030 – 1032.
[5] W. Y. Wu, C. Mee, F. Califano, R. Banki, D. W. Wood, *Nat. Protoc.* **2006**, *1*, 2257 – 2262.
[6] D. Meyer, A. Chilkoti, *Nat. Biotechnol.* **1999**, *17*, 1112 – 1115.
[7] Y. Cho, Y. Zhang, T. Christensen, L. B. Sagle, A. Chilkoti, P. S. Cremer, *J. Phys. Chem. B* **2008**, *112*, 13765 – 13771.
[8] M. R. Dreher, A. J. Simnick, K. Fischer, R. J. Smith, A. Patel, M. Schmidt, A. Chilkoti, *J. Am. Chem. Soc.* **2008**, *130*, 687 – 694.
[9] W. Hassouneh, T. Christensen, A. Chilkoti, *Curr. Protoc. Protein Sci.* **2010**, *61*, 6.11.1 – 6.11.16.

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[10] X. Ge, D. S. Yang, K. Trabbic-Carlson, B. Kim, A. Chilkoti, C. D. Filipe, *J. Am. Chem. Soc.* **2005**, *127*, 11228 – 11229.
[11] M. R. Banki, L. Feng, D. W. Wood, *Nat. Methods* **2005**, *2*, 659 – 661.
[12] U. Ilangovan, H. Ton-That, J. Iwahara, I. Schneewind, R. T. Clubb, *Proc. Natl. Acad. Sci. USA* **2001**, *98*, 6056 – 6061.
[13] R. G. Kruger, B. Otvos, B. A. Frankel, M. Bentley, P. Dostal, D. G. McCafferty, *Biochemistry* **2004**, *43*, 1541 – 1551.
[14] P. Ameloot, W. Declercg, W. Fiers, P. Vandenabeele, P. Brouckaert, *J. Biol. Chem.* **2001**, *276*, 27098 – 27103.
[15] X. M. Zhang, I. Weber, M. J. Chen, *J. Biol. Chem.* **1992**, *267*, 24069 – 24075.
[16] H. J. Schoenfeld, B. Poeschl, J. R. Frey, H. Loetscher, W. Hunziker, A. Lustig, M. Zulauf, *J. Biol. Chem.* **1991**, *266*, 3863 – 3869.
[17] D. A. Flick, G. E. Gifford, *J. Immunol. Methods* **1984**, *68*, 167 – 175.
[18] J. D. Liddil, R. T. Dorr, P. Scuderi, *Cancer Res.* **1989**, *49*, 2722 – 2728.
[19] H. Mao, S. A. Hart, A. Schink, B. A. Pollok, *J. Am. Chem. Soc.* **2004**, *126*, 2670 – 2671.
[20] J. M. Antos, G. M. Miller, G. M. Grotenbreg, H. L. Ploegh, *J. Am. Chem. Soc.* **2008**, *130*, 16338 – 16343.
[21] J. M. Antos, G. L. Chew, C. P. Guimaraes, N. C. Yoder, G. M. Grotenbreg, M. W. Popp, H. L. Ploegh, *J. Am. Chem. Soc.* **2009**, *131*, 10800 – 10801.
[22] Z. Wu, X. Guo, Q. Wang, B. M. Swarts, Z. Guo, *J. Am. Chem. Soc.* **2010**, *132*, 1567 – 1571.
[23] M. W. Popp, S. K. Dougan, T. Y. Chuang, E. Spooner, H. L. Ploegh, *Proc. Natl. Acad. Sci. USA* **2011**, *108*, 3169 – 3174.
[24] S. C. Gill, P. H. von Hippel, *Anal. Biochem.* **1989**, *182*, 319 – 326.

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