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ARTICLE

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# Engineering of heterobifunctional biopolymers for tunable binding and precipitation of Strep-Tag proteins and virus-like nanoparticles

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## Abstract

Affinity precipitation is a powerful separation method in that it combines the binding selectivity of affinity chromatography with precipitation of captured biomolecules via phase separation triggered by small changes in the environment, e.g., pH, ionic strength, temperature, light, etc. Elastin-like polypeptides (ELPs) are thermally responsive biopolymers composed of pentapeptide repeats VPGVG that undergo reversible phase separation, where they aggregate when temperature and/or salt concentration are increased. Here we describe the generation of an ELP fusion to a soluble streptavidin mutant that enables rapid purification of any *Strep*-tag II fusion protein of interest. This heterobifunctional protein takes advantage of the native tetrameric structure of streptavidin, leading to binding-induced multivalent cross-linking upon protein capture. The efficient biotin-mediated dissociation of the bound *Strep*-tag II fusion protein from the streptavidin-ELP capturing scaffold allows for mild elution conditions. We also show that this platform is particularly effective in the purification of a virus-like particle (VLP)-like E2 protein nanoparticle, likely because the high valency of the protein particle causes binding-induced crosslinking and precipitation. Considering the importance of VLP for gene therapy applications, we believe this is a particularly exciting advance. We demonstrated this feasibility by the efficient purification of a VLP-like E2 protein nanoparticle as a surrogate.

**KEYWORDS**
Affinity purification, ELP, Strep tag

## 1 | INTRODUCTION

The affinity system based on engineered tetrameric streptavidins, known as Strep-Tactin® and Strep-TactinXT® and its cognate peptide binding partner, *Strep*-tag® II, is a powerful platform that allows for the purification, biomolecular labeling, immobilization, and functional elucidation of a diverse set of recombinant proteins (Ayala et al., 2013; Guo et al., 2023; Johar & Talbert, 2017). Compared to the conventional polyhistidine-tag system (His-tag), which is primarily utilized in the purification of recombinant proteins through immobilized metal ion affinity chromatography (Bornhorst & Falke, 2000; Stiborova et al., 2003), Strep-tags offer a more versatile solution as demonstrated by their efficacy in other applications, including the isolation of exosomes (Guo et al., 2023) and organelles (Xiong et al., 2019) with high purity from cell culture supernatants, enrichment of chimeric antigen receptor (CAR) and T cell receptor (TCR)-engineered T cells via flow cytometry (Liu et al., 2016), and the isolation and purification of G-protein-coupled receptors (Yeliseev et al., 2017).

**Abbreviations:** ELPs, elastin-like-polypeptides; Streptavidin, Strep; Strep-Tag II, ST2.

  

However, a significant challenge that precludes its broader use on a large-scale basis is the limited soluble expression and preparation of the actual protein. While significant steps have been taken to improve the production of wild-type streptavidin in engineered microorganisms (Markwick et al., 2003; Nagarajan et al., 1993; Nogueira et al., 2014), it is still challenging to express soluble streptavidin in the most commonly used expression host, *Escherichia coli* (Sano & Cantor, 1990). Most of the expressed streptavidin forms inclusion bodies, which requires both denaturation and in vitro refolding of the protein, a costly process that can take several days to complete, and results in limited total yield. In an effort to improve the solubility of streptavidin, several groups have tested a variety of fusion tags, including the translation initiation factor IF2 (Sørensen et al., 2003) and T7-peptide tag (Gallizia et al., 1998), and saw significant improvements in solubility. However, the expression levels and purity were highly variable, and still required chromatographic capturing technologies, which could inevitably present a significant technical challenge to those who are less experienced and creates a barrier to its broader application.

Another challenge preventing the broader utility of *Strep*-tag for organelle and cell isolation is the use of expensive affinity resins based on immobilized streptavidin (Schmidt & Skerra, 1994), which can limit the overall throughput and process scale-up. This bottleneck has generated significant interest in non-chromatographic alternatives as a new capture platform. Our group has demonstrated the feasibility of employing a wide range of elastin-like polypeptide (ELP)-fusion proteins for affinity purification by taking advantage of its well-characterized inverse temperature cycling (ITC) property (Lao et al., 2006; Liu et al., 2012; Madan et al., 2013; Swartz & Chen, 2018). In one example, ELP was tethered to a small (7 kDa) antibody-binding Z-domain (Z-ELP) for monoclonal antibody (mAb) purification (Madan et al., 2013). Improved mAb recovery without any salt addition was made possible by conjugating Z-ELP to a 25 nm, 60-mer E2 protein nanocage (Z-ELP-E2) to increase the dimensionality and valency of binding, resulting in spontaneous mAb-induced crosslinking and precipitation (Swartz et al., 2017). That streptavidin is naturally tetravalent may enable a similar affinity purification scheme based on target protein-induced crosslinking and aggregation.

To that end, we demonstrate here the highly soluble expression of two streptavidin variants, Strep-Tactin (Voss & Skerra, 1997) and Strep-TactinXT (Schmidt et al., 2021), by inserting a T7 peptide tag to the N-terminus. To simplify purification and its use as an affinity agent for purification of *Strep*-tag fused ligands, ELP was fused to the C-terminus. By combining the tunable phase-separation properties of ELP and binding selectivity of *Strep*-tag/streptavidin, we demonstrate that a wide range of Strep-tag II (ST2) fusion proteins, ranging from monomeric and dimeric to multimeric, can be captured either by ELP-induced phase separation or by induced crosslinking upon binding (Figure 1). Captured proteins were eluted under physiological conditions by the addition of biotin and highly pure proteins were obtained by removing Strep-Tactin-ELP (Strep-ELP80) or Strep-TactinXT-ELP (StrepXT-ELP80) by ITC.

In the current surge of interest in developing vectors for gene therapy such as adeno-associated virus (Wang et al., 2024), chromatography is again the dominant unit operation, despite the very low capacities resulting from the limited access of the virus to the resin pore space (Trilisky & Lenhoff, 2007). In contrast, the ligand/target combination of the multivalent StrepTactin together with a large, multivalent target is especially well suited to affinity precipitation. We therefore present a proof-of-concept lab-scale experimental scheme using the Strep-ELP80 and a ST2-tagged E2 nanocage as a surrogate for the affinity purification of a virus-like particle (VLP). We believe that this platform will enable a simple, direct purification and recovery of a variety of *Strep*-tag fusion proteins and nanostructures, obfuscating the need for expensive chromatographic resins and dramatically lowering the processing time and material consumption.

## 2 | MATERIALS AND METHODS

### 2.1 | Materials

*Escherichia coli* strain BLR(DE3) containing pET24(a) vectors encoding for Strep-ELP[$KV_8F$-80]-SpyCatcher and StrepXT- ELP[$KV_8F$-80]-SpyCatcher. *E. coli* strain BL21(DE3) containing a pET11(a) vector encoding for ST2-E2(158). pDEST14-SpyCatcher was a gift from Mark Howarth (Addgene plasmid # 35044). pET11(a) vectors containing the E2 core subunit (179–427) with various native N-terminal extensions at amino acid sites ranging from position 152–179 (pE2–152, pE2–158, pE2–167, pE2–173, and pE2–179) were received as a gift from Prof. Szu-Wen Wang (Dalmau et al., 2008). Bacto tryptone and yeast extract were purchased from BD Biosciences (Franklin Lakes, NJ). Kanamycin, ampicillin, bovine serum albumin (BSA), and isopropyl-β-D-thiogalactoside (IPTG) were purchased from Fisher Scientific (Pittsburgh, PA). Sodium hydroxide, sodium phosphate, phosphate buffered saline (PBS) tablets, α-lactalbumin, citric acid, tris base, ammonium sulfate (AS), and sodium chloride were purchased from Sigma-Aldrich (St. Louis, MO). Buffer BXT (10x) was purchased from IBA Life Sciences (Göttingen, Germany). 96-well solid black plates were purchased from Corning (Corning, NY). The synthetic *Strep*-tag II peptide with sequence 2-Abz-SAWSHPQFEK-$NH_2$ was purchased from GenScript (Piscataway, NJ). Precast SDS-PAGE gels were purchased from Bio-Rad (Hercules, CA).

### 2.2 | Genetic manipulations and vector construction

*E. coli* strain NEB 5-alpha (NEB #C29871) was used as the host for all genetic manipulations. All ELP constructs were cloned in pET24(a) vectors, and all *Strep*-tag II-E2 constructs were cloned in pET11(a) vectors. Strep- and StrepXT-$ELP_{80}$-SpyCatcher was constructed by

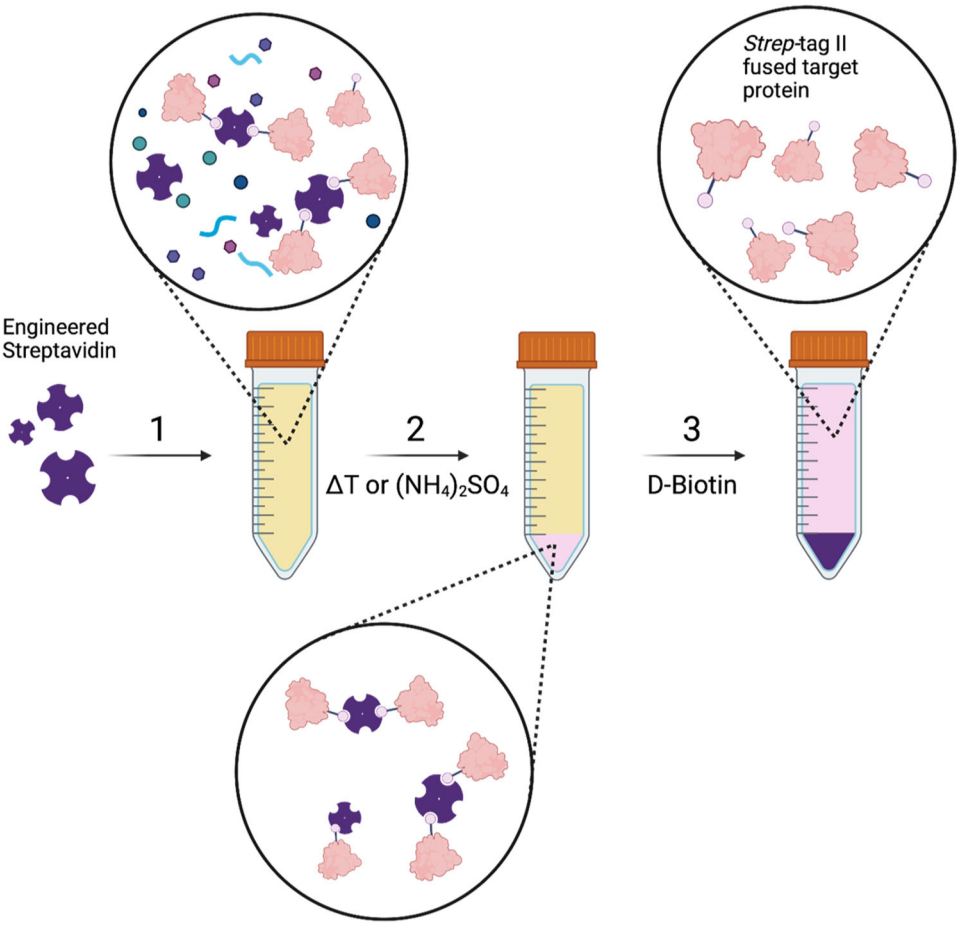


**FIGURE 1** A schematic of the overall purification process. Enhanced affinity precipitation of Strep-tag II fusion proteins using engineered streptavidin constructs through a combination of increased aggregate sizes and cross-linking via multivalent interactions. Step 1: Direct mixing of Strep/StrepXT-ELP fusion proteins inxto cell lysates containing target protein of interest. Step 2: Multivalent binding between the tetrameric streptavidin constructs and Strep-tag II target proteins result in cross-linking and formation of an insoluble precipitate following an increase in temperature or salt concentration. Step 3: Dissociation and resolubilization of both Strep-tag II target proteins and Strep/StrepXT-ELP in mild buffer conditions containing excess D-Biotin.

**TABLE 1** Constructs used in this study.

| Strep-ELP80 | Streptactin fused to ELP[$KV_8F$-80]-SpyCatcher domain |
|---|---|
| StrepXT-ELP80 | Engineered streptactin fused to ELP[$KV_8F$-80]-SpyCatcher domain |
| Z-ELP80 | Variant of B-domain from Staphylococcal protein A fused to ELP[$KV_8F$-80]-SpyCatcher |
| ST2-mRuby | *Strep*-Tag II-fused mRuby |
| ST2-yCD | *Strep*-Tag II-fused yeast cytosine deaminase |
| ST2-E2 | *Strep*-Tag II-fused 60-mer E2 protein nanocage |

PCR of the Strep-Tactin and Strep-TactinXT gBlock where the first 14 amino acid sequence was replaced by the T7-tag (IDT, Coralville, Iowa). The PCR-amplified products were then digested with NcoI and EcoRI and ligated into NcoI- and EcoRI-digested ELP[$KV_8F$-80]-SpyCatcher. ST2-E2 constructs were generated by annealing ST2 (SAWSHPQFEK) phosphorylated oligos using pE2-158 as the backbone template following blunt-end ligation. All constructs used in this study are summarized in Table 1.

## 2.3 | Protein expression and purification

All ELP-fusion constructs were expressed in BLR(DE3) *E. coli* grown in autoinduction media (AIM) with 100 μg/mL kanamycin at 30°C and 250 rpm for 24 h and were purified by inverse transition cycling (ITC) using 0.5 M ammonium sulfate, as described previously (Swartz et al., 2018b). The ST2-mRuby and ST2-yCD proteins were expressed in BLR(DE3) *E. coli* grown in Terrific Broth (TB) with 50 μg/mL kanamycin at 37°C and 250 rpm for 24 h with leaky expression. The purified ELP-fusion concentration was estimated by Bradford protein assay purchased from Bio-Rad (Hercules, CA) using BSA as a standard. ST2-E2 constructs were expressed in BL21(DE3) *E. coli* cells grown in LB with 100 μg/mL ampicillin at 37°C and 250 rpm until an OD600 of 0.5, where the culture was induced with 0.2 mM IPTG at 20°C for 20 h. After protein expression, all cultures were harvested

by centrifugation at 4000 × *g* for 15 min at 4°C and resuspended in a TN150 buffer (50 mM Tris, 150 mM sodium chloride, pH 8.0). Cells were lysed using a Fisher Sonicator (Pittsburgh, PA) using 5 s pulse on and 10 s pulse off for 5 min over ice. All E2 constructs were partially purified by incubating at 70°C for 10 min and centrifugation at 15,000 × *g* for 15 min to isolate the soluble proteins. The soluble E2 sample was filtered through a 0.8/0.2 µm Supor Acrodisc syringe filter. Protein expression was confirmed by Coomassie-stained, 10% acrylamide SDS-PAGE using a Bio-Rad Mini-PROTEAN electrophoresis system (Hercules, CA). Protein purity was estimated using densitometry analysis of SDS-PAGE gels using Thermo MyImage software (Waltham, MA).

### 2.4 | Fluorescence titration

Fluorescence titration was performed as previously reported with slight modifications (Voss & Skerra, 1997). Briefly, titration experiments were carried out with a BioTek Synergy H4 Plate Reader that was thermostated at 25°C. Wavelengths for excitation and emission were set to 280 and 340 nm, respectively, with slit widths of 5 nm. A 200 µL volume of a 1 µM Strep-ELP80 and StrepXT-ELP80 solution in 50 mM Tris, 150 mM sodium chloride, pH 8.0, was pipetted into a black 96-well plate and thermally equilibrated. Then 0.4–1.6 µL aliquots of a 10 µM–1 mM solution (10 µL in total) of the peptide in the same buffer, which had been prepared gravimetrically, were added, and mixed with the mixing setting on the plate reader for 5 min and the fluorescence intensity was measured. The slight volume increase during the titration (5%) was neglected. The data were fitted by nonlinear least-squares regression.

### 2.5 | Batch target protein capture and purification

All target proteins (ST2-mRuby, ST2-yCD, and ST2-E2) were prepared as described above. After protein expression, all cultures were harvested by centrifugation at 4000 × *g* for 15 min at 4°C and resuspended in a TN150 buffer (50 mM Tris, 150 mM sodium chloride, pH 8.0). Cells were lysed using a Fisher Sonicator (Pittsburgh, PA) using 5 s pulse on and 10 s pulse off for 5 min over ice. The cell lysate was spun down at 10,000 × *g* for 15 min at 4°C, and the supernatant was collected without any further purification and used for downstream capture using the Strep-ELP80 and StrepXT-ELP80 system.

Purified Strep/StrepXT-ELP80 were directly mixed with cell lysates containing the target protein at a 1:1, 2:1, 4:1 molar ratio in triplicate for 30 min at 4°C, then pelleted using 0.3 M ammonium sulfate and centrifuged at 16,000 × *g* for 10 min at 25°C. The pelleted Strep/StrepXT-ELP80 – target protein complex was resuspended in 1x BXT elution buffer (100 mM Tris/HCl, pH 8.0, 150 mM NaCl, 1 mM EDTA, 50 mM biotin) and mixed for 30 min at 4°C. The elution samples were adjusted to 0.3 M ammonium sulfate or warmed to 37°C for selective Strep/StrepXT-ELP80 precipitation. The purified target protein was removed in the supernatant, and the target protein elution yield was calculated by measuring absorbance by using the Bradford assay and densitometry analysis of SDS-PAGE gels.

## 3 | RESULTS AND DISCUSSION

### 3.1 | Construction and expression of capturing Strep-Tactin fusion proteins using the truncated, mutagenized core streptavidin domain

Mature streptavidin consists of 159 amino acids (Argarana et al., 1986) but full-length, nontruncated streptavidin molecules are rarely observed under typical *Streptomyces avidinii* culture conditions due to their extreme susceptibility to proteolysis at the terminal regions, and both full-length and partially truncated streptavidin are prone to form higher-order aggregates and have poor solubility (Pähler et al., 1987; Sano et al., 1995). In contrast, fully truncated core streptavidins exhibit excellent solubility and minimal aggregation (Argarana et al., 1986; Pähler et al., 1987). To further enhance solubility, the truncated core of two streptavidin variants, Strep-Tactin and Strep-TactinXT, was generated by deletion of the first 14 amino acids (1–159 of the streptavidin sequence) and replaced by a T7-tag peptide, followed by fusion to an ELP[$KV_8F$]80 domain at the C-terminus. Expression of both Strep-ELP80 and StrepXT-ELP80 was under the control of a strong T7 promoter. By exploiting the unique phase transition behavior of ELPs, the fusion protein can be separated from soluble contaminants by triggering the phase transition of ELP (Figure 2) by increasing the salt concentration to 0.5 M ammonium sulfate following clarification of the cell lysate. In subsequent rounds of ITC, Strep/StrepXT-ELP80 was warmed to room temperature and was able to form insoluble aggregates without the addition of salt, most likely due to its propensity to form tetramers, and thereby simplifying purification. Purified proteins were analyzed by SDS-PAGE and only a single band of the expected protein was detected in both cases. Roughly 18 mg/L and 15.8 mg/L of the Strep-ELP80 and StrepXT-ELP80 fusion protein, respectively, was obtained.

### 3.2 | Purification of a monomeric fusion target protein

Our initial experiments were conducted using Strep-ELP80, where the exposed, flexible loop formed by the streptavidin residues 44–47 was mutated from ESAV to VTAR to improve ST2 binding, with a reported $K_d$ value of 1.4 µM (Voss & Skerra, 1997). While our initial protein purification experiments using Strep-ELP80 were successful in recovering a monomeric *Strep*-tag II mRuby (ST2-mRuby) fusion protein directly from cell lysate, the overall yield was much lower than the expected 1:1 binding stoichiometry (Figure 3). The two product bands of mRuby in lanes 6 and 10 correspond to imine hydrolysis of the chromophore after boiling during sample preparation for SDS-PAGE. Unexpectedly, a small amount of ST2-mRuby was

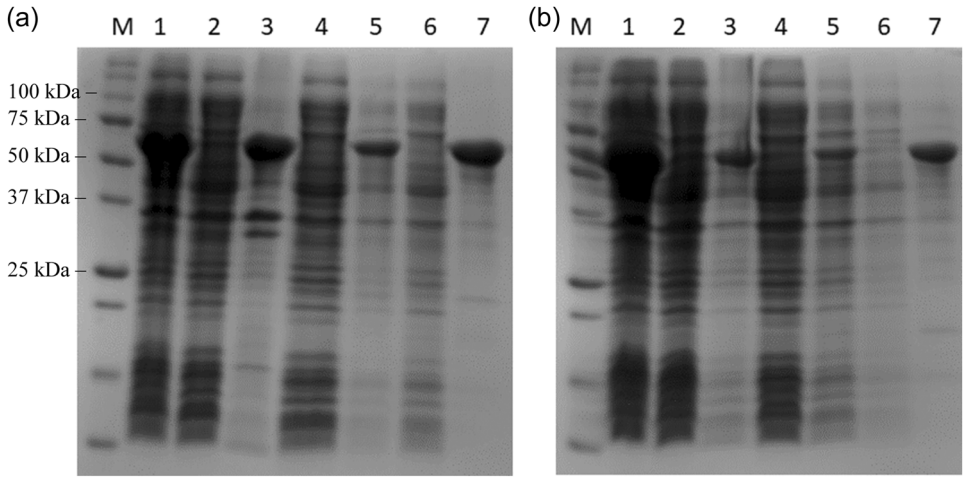


**FIGURE 2** SDS-PAGE following the purification of (**a**) Strep-ELP80 and (**b**) StrepXT-ELP80 stained with Coomassie blue. Lane 1 on both gels: cell lysate after sonication; Lane 2: soluble cell lysate; Lane 3: insoluble fraction following cell lysis; Lane 4: the supernatant fraction following the first hot spin; Lane 5: the supernatant after resuspension of the hot spin pellet; Lane 6: the supernatant from the final hot spin. Lane 7: resuspension of the hot spin pellet in Tris buffer. The molecular weights of Strep-ELP80 and StrepXT-ELP80 are 61.3 kDa and 59.2 kDa, respectively.

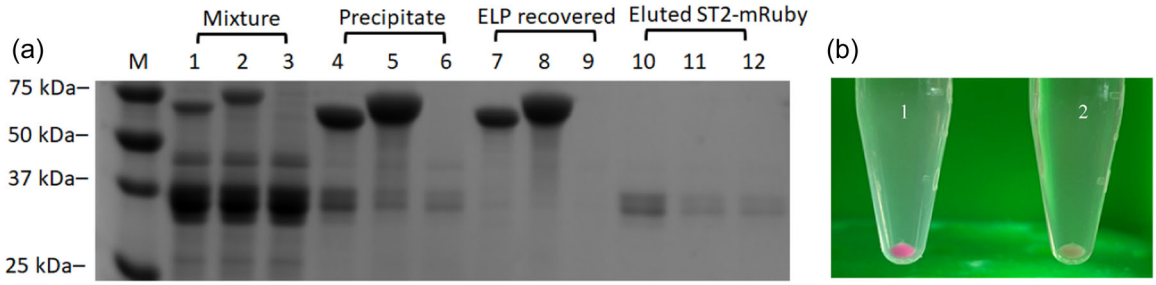


**FIGURE 3** Strep-tag II fused mRuby (ST2-mRuby) affinity precipitation from cell lysates using Strep-ELP80 and Z-ELP80 as a negative control. (**a**) SDS-PAGE analysis of ST2-mRuby purification from cell lysate using either Strep-ELP80 (Lane 1, 4, 7, 10), Z-ELP80 (Lane 2, 5, 8, 11), or no ELP (Lane 3, 6, 9, 12) and 0.3 M ammonium sulfate for precipitation. Lane 1: Strep-ELP80 and ST2-mRuby after 30 min incubation at 4°C. Lane 2: Z-ELP80 and ST2-mRuby after 30 min incubation at 4°C. Lane 3: ST2-mRuby only. Lane 4: Strep-ELP80 and ST2-mRuby after precipitation and resuspension in elution buffer. Lane 5: Z-ELP80 and ST2-mRuby after precipitation and resuspension in elution buffer. Lane 6: ST2-mRuby cell lysate only after addition of 0.3 M ammonium sulfate and addition of elution buffer. Lane 7: Precipitated Strep-ELP80 following elution and collection of ST2-mRuby. Lane 8: Precipitated Z-ELP80 following elution and collection of ST2-mRuby. Lane 9: ST2-mRuby (no precipitation). Lane 10: Collected supernatant following elution and dissociation of Strep-ELP80 and ST2-mRuby complex. Lane 11: Collected supernatant following elution and dissociation of Z-ELP80 and ST2-mRuby complex. Lane 12: Following same elution and collection conditions for ST2-mRuby only. The expected molecular weight of ST2-mRuby is 27.7 kDa. (**b**) A visual comparison between captured ST2-mRuby in Strep-ELP80 (1, left) and Z-ELP80 (2, right) after precipitation.

observed after precipitation even without any ELP (Lane 6), indicative of partial aggregation under the precipitation condition.

This lower recovery was not the result of inefficient elution as no ST2-mRuby (10 μM) was detected in the final recovered Strep-ELP80 fraction. Rather, it is likely due to a lower binding affinity of the ELP fusion as confirmed by a measured $K_d$ value of 2.4 μM (Figure 4). Additionally, the expression of soluble, active streptavidin in the cytosol has been reported to lead to depletion of the host cell's biotin pool, and as a result, reduces the number of binding sites in Strep-ELP80 due to its high affinity for biotin.

The lower binding affinity of Strep-ELP led us to find an alternative streptavidin mutant with enhanced binding affinity towards the *Strep*-tag II peptide. Recently, crystal structures of the streptavidin mutant revealed that the loop comprised of residues 115-121 could offer additional contacts with the *Strep*-tag II peptide bound to the opposite streptavidin subunit. The A117E, W120G and K121Y substitutions (in the context of the optimized loop 44-47 of streptavidin) showed a 5- to sixfold improved binding affinity as compared to the Strep-Tactin mutant, with a $K_d$ value of 75 nM (Schmidt et al., 2021). Following fusion of the ELP80 domain, we obtained a $K_d$ value of 390 nM (Figure 4), which was sixfold better than our Strep-ELP80 fusion and comparable to the difference in fold-change reported previously. This mutant (StrepXT-ELP80) was subsequently used in further studies.

We next investigated whether the enhanced binding affinity of StrepXT-ELP80 could improve the capturing efficiency of ST2-mRuby. We first incubated StrepXT-ELP80 with *E. coli* cell lysates containing ST2-mRuby for 30 min at 4°C. Following precipitation, roughly 50% recovery was observed using a 1:1 StrepXT-ELP80 to ST2-mRuby molar ratio (Figure 5). Close to 100% recovery was detected by increasing the molar ratio to 4:1. Using StrepXT-ELP80 significantly improved the binding capacity of the ST2-mRuby construct, surpassing that of Strep-ELP80, particularly at molar ratios of 2:1 (Figure 5). These findings indicate a substantial improvement in the capturing efficacy of the target protein when utilizing the StrepXT-ELP80 construct.

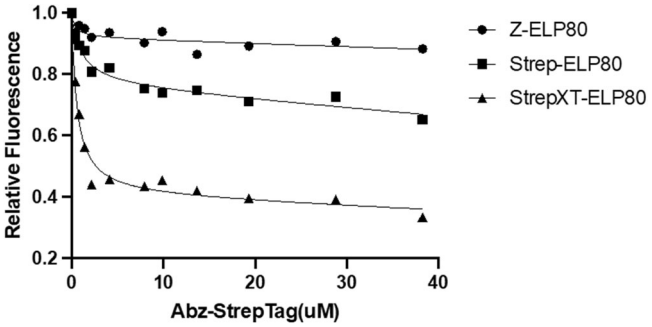


**FIGURE 4** Measurement of the affinity between the Strep-tag II, Strep-ELP80, StrepXT-ELP80 and a control. Binding isotherms from fluorescence titration experiments with Strep-ELP80, StrepXT-ELP80, Z-ELP80 and the synthetic Abz-coupled Strep-tag II peptide. Relative fluorescence intensities were plotted against the total peptide concentration for 'Z-ELP80' (circles), 'Strep-ELP80' (squares), and 'StrepXT-ELP80' (triangles).

## 3.3 | Enhanced capture by multivalent target-streptavidin interactions

As previously reported, a significant rise in affinity (by a factor of ~10) can be observed when streptavidin is titrated with a bivalent Twin-Strep-tag (Schmidt et al., 2021). This finding underscores the biophysical nature of the avidity effect, which is independent of the specific strength and mechanism of interaction at individual binding sites. Crystal structure analysis further reveals that the Twin-Strep-tag peptide is capable of spanning two binding sites on a single face of the streptavidin homo-tetramer, providing a structural explanation for the remarkable avidity effect observed in binding experiments. Here, we asked if similar bivalent interactions can be observed in the presence of multivalent proteins, and whether or not protein retention could be improved by multivalent affinity ligand interactions.

To test this possibility, we used StrepXT-ELP80 in the recovery of a dimeric yeast cytosine deaminase (yCD) directly from cell lysate. This enzyme is of great clinical interest as it possesses the ability to convert the nontoxic prodrug 5-fluorocytosine (5-FC) into the chemotherapeutic agent 5-fluorouracil (5-FU) (Hartzell et al., 2020; Lieser et al., 2019; Lieser et al., 2020). Intriguingly, our observations revealed an exceptional capture extent approaching 100% (as determined through densitometry analysis), indicating a bivalent mode of binding. This is consistent with the avidity effect, which occurs when the *Strep*-tag II presenting dimeric yCD simultaneously interacts with two binding sites on the same StrepXT tetramer (Figure 6). This is reflected by the 100% recovery using a molar ratio of 2:1, suggesting that the higher valency of the dimeric yCD offers the benefit of significantly tighter binding, with a reduced off-rate, while maintaining the inherent reversibility of the ligand-receptor interaction following elution with biotin as a competing ligand.

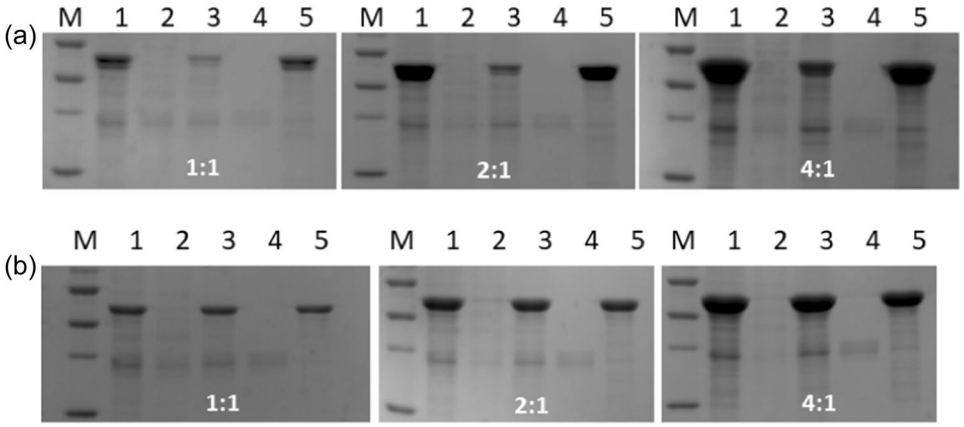


**FIGURE 5** SDS-PAGE analysis of binding efficiencies between Strep-ELP80 and StrepXT-ELP80 using Strep-tag II fused mRuby (ST2-mRuby) as a target protein with varying 1:1, 2:1, and 4:1 Strep:mRuby molar ratios. (**a**) Lane 1: Cell lysate containing ST2-mRuby and the addition of Strep-ELP80. Lane 2: Collected supernatant following co-precipitation of the Strep-ELP80 and ST2-mRuby complex. Lane 3: Resuspension and elution of the Strep-ELP80 and ST2-mRuby precipitated complex. Lane 4: Collected supernatant after precipitation of Strep-ELP80. Lane 5: Resuspension of precipitated Strep-ELP80. (**b**) Lane 1: Cell lysate containing ST2-mRuby and the addition of StrepXT-ELP80. Lane 2: Collected supernatant following co-precipitation of the StrepXT-ELP80 and ST2-mRuby complex. Lane 3: Resuspension and elution of the StrepXT-ELP80 and ST2-mRuby precipitated complex. Lane 4: Collected supernatant after precipitation of StrepXT-ELP80. Lane 5: Resuspension of precipitated StrepXT-ELP80.

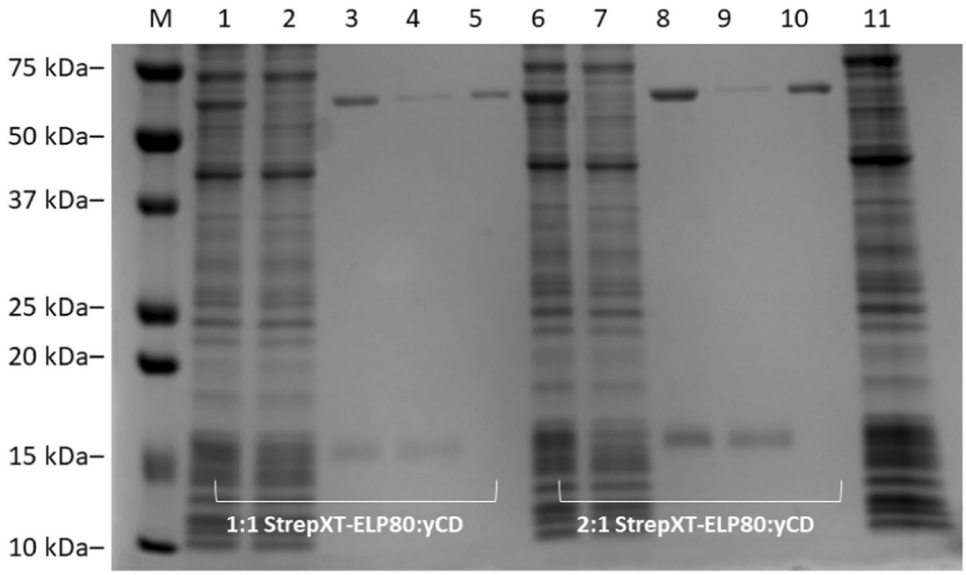


**FIGURE 6** Affinity precipitation from cell lysates containing the dimeric Strep-tag II yCD (ST2-yCD) fusion protein using StrepXT-ELP80 at 1:1 (Lane 1-5) and 2:1 (Lane 6-10) molar ratios. Lane 1: Cell lysate containing ST2-yCD and the addition of StrepXT-ELP80. Lane 2: Collected supernatant following co-precipitation of the StrepXT-ELP80 and ST2-yCD complex. Lane 3: Resuspension and elution of the StrepXT-ELP80 and ST2-yCD precipitated complex. Lane 4: Collected supernatant after precipitation of StrepXT-ELP80. Lane 5: Resuspension of precipitated StrepXT-ELP80. Lane 6-10 are the same as Lane 1-5, except experiments were conducted at a 2:1 StrepXT-ELP80:ST2-yCD molar ratio. Lane 11: ST2-yCD cell lysate control.

However, it should be noted that this tighter binding resulted in a small population of irreversibly bound StrepXT-yCD complexes, as noted by the presence of StrepXT-ELP80 in lanes 4 and 9.

## 3.4 | Efficient purification of VLP-like particles by multivalent crosslinking and precipitation

In addition to the interactions with the same homotetrameric face of StrepXT-ELP80, crosslinking with multiple StrepXT tetramers is also possible for larger multimeric proteins such as VLPs, which are being increasingly used for gene therapy applications (Wang et al., 2024). The feasibility of exploiting binding-induced crosslinking has been demonstrated by using the bivalent interaction between mAbs and Z domain-decorated protein nanocages to significantly improve mAb precipitation and recovery (Swartz & Chen, 2018; Swartz et al., 2018a). To investigate whether similar behavior could be achieved using the tetrameric StrepXT, we inserted a *Strep*-tag II to the N-terminus of a 25 nm, 60-mer E2 protein nanocage (ST2-E2) as a surrogate to natural VLPs (Chen et al., 2015; Sun et al., 2015). An immediate increase in turbidity was observed upon mixing StrepXT-ELP80 with ST2-E2, indicative of crosslinking. As expected, we were able to capture 100% of ST2-E2, following incubation for 30 min and centrifugation. Interestingly, after elution of ST2-E2 with D-biotin, we observed significant retention ( > 50%) of the ST2-E2 in the precipitant (Figure 7).

A second elution following the same resuspension and co-precipitation procedure did not reduce the amount of ST2-E2 bound to StrepXT-ELP80 (Figure 7, lane 5, 6, 7), even when the elution steps were performed in the presence of excess D-biotin. It is possible that the

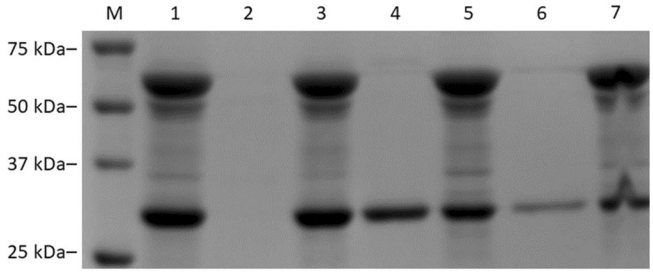


**FIGURE 7** Affinity precipitation from cell lysates containing the 60-mer Strep-tag II E2 (ST2-E2) fusion protein using StrepXT-ELP80 with sequential elution steps. Lane 1: Cell lysate containing ST2-E2 and the addition of StrepXT-ELP80. Lane 2: Collected supernatant following co-precipitation of the StrepXT-ELP80 and ST2-E2 complex. Lane 3: Resuspension and elution of the StrepXT-ELP80 and ST2-E2 precipitated complex. Lane 4: Collected supernatant after precipitation of StrepXT-ELP80. Lane 5: Another round of resuspension of precipitated StrepXT-ELP80 and ST2-E2 in elution buffer from previous step. Lane 6: Collected supernatant after precipitation of StrepXT-ELP80. Lane 7: Resuspension of the StrepXT-ELP80 and ST2-E2 precipitated complex.

accumulated binding strength of the multiple StrepXT-ST-E2 interactions can be several orders of magnitude higher than that of the StrepXT-biotin ($K_d = 10^{-14}$ M) interaction (Michael Green, 1990), resulting in incomplete elution and dissociation of the StrepXT-ST-E2 complex.

Next, we investigated whether the avidity effect could be tuned by employing the Strep-ELP80 variant, which possesses a lower binding affinity for Strep-tag II. Remarkably, 100% capture of ST2-E2 was still observed using the same incubation and co-precipitation procedures, again highlighting the advantage of multivalent binding

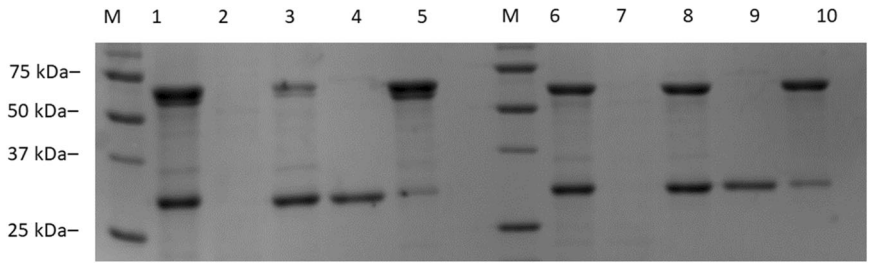


**FIGURE 8** Affinity precipitation from cell lysates containing the 60-mer Strep-tag II E2 (ST2-E2) fusion protein using Strep-ELP (Lane 1-5) and StrepXT-ELP80 (Lane 6-10) Lane 1: Cell lysate containing ST2-E2 and the addition of Strep-ELP80. Lane 2: Collected supernatant following co-precipitation of the Strep-ELP80 and ST2-E2 complex. Lane 3: Resuspension and elution of the Strep-ELP80 and ST2-E2 precipitated complex. Lane 4: Collected supernatant after precipitation of Strep-ELP80. Lane 5: Resuspension of precipitated Strep-ELP80 and ST2-E2 in elution buffer. Lane 6-10 are the same as Lane 1-5, except experiments were conducted with StrepXT-ELP80.

on improving protein purification. However, the real benefit of utilizing Strep-ELP80 is the improved elution, as 80% of the bound ST2-E2 was successfully recovered upon elution, in stark contrast to the 50% recovery achieved when employing StrepXT-ELP80 (Figure 8). These findings emphasize the significant impact of utilizing different variants on the overall efficacy of the avidity effect.

## 4 | CONCLUSIONS

This study demonstrates substantial advances compared to conventional resin-based column chromatography methods. The implementation of a one-step binding and precipitation technique enables rapid and specific pulldown of any Strep-tag II fused proteins directly from cell lysates and complex media components. Following precipitation, the Strep/StrepXT-ELP80-ST2 target protein complex can be re-suspended and washed in physiological buffers and eluted in the presence of excess biotin. Upon elution, a selective precipitation step employing a low salt concentration as low as 0.1 M effectively separates the Strep/StrepXT-ELP80 from the target protein. Similarly, Strep/StrepXT-ELP80 can be regenerated using 0.05 M NaOH, which removes the biotin from the binding pocket. These same principles can also be applied in a continuous manner using synthetic membranes.

Moreover, the Strep/StrepXT-ELP80 system can be extended to various applications beyond its traditional role in protein purification. One notable application is the isolation of live, whole cells. By genetically fusing the Strep-tag to an exposed cell surface protein or a protein of interest, researchers can isolate a population of cells within a heterogeneous mixture of cells, simply by the addition of the Strep/StrepXT-ELP80 protein to the cell media. Given the overall size of cells (in comparison to individual proteins), selective pulldowns of the intended cellular target would, in theory, be much easier to achieve. Additionally, the specific and reversible interaction between streptavidin and the Strep-tag allows for efficient and stable immobilization of proteins onto various solid supports, enabling sensitive detection and analysis of molecular interactions. Overall, our Strep/StrepXT-ELP80 system presents a versatile toolset for applications that could encompass protein tracking, cell isolation, and surface functionalization, extending its utility beyond protein purification.

### AUTHOR CONTRIBUTIONS

James Tang, Abraham Lenhoff and Wilfred Chen conceived the project; James Tang, Abraham Lenhoff, Matthew Becker and Wilfred Chen designed experiments; James Tang and Matthew Becker performed the experiments; James Tang, Abraham Lenhoff, Matthew Becker and Wilfred Chen analyzed the data, and James Tang, Abraham Lenhoff, Matthew Becker and Wilfred Chen wrote the paper. All authors discussed the results and commented on the manuscript.

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### CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interests.

### DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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