SpyTag/SpyCatcher Functionalization of E2 Nanocages with Stimuli-Responsive Z-ELP Affinity Domains for Tunable Monoclonal Antibody Binding and Precipitation Properties

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Supporting Information

ABSTRACT: E2 nanocages functionalized with Z-domain-elastin-like polypeptide affinity ligands (Z-ELP40) using Sortase A (SrtA) ligation have been shown to be a promising scaffold for purifying monoclonal antibodies (mAbs) based on affinity precipitation. However, the reversible nature of SrtA reaction has been attributed to the low ligation efficiency (<25%) and has significantly limited the practical utility of the technology. Here, we reported an improved conjugation platform using the SpyTag/SpyCatcher pair to form a spontaneous isopeptide bond between SpyTag-E2 and Z-ELP-SpyCatcher fusion proteins of two different ELP chain-lengths. Using this system, E2 ligation efficiencies exceeding 90% were obtained with both 40- and 80-repeat Z-ELP-SpyCatcher fusions. This enabled the production of nanocages fully functionalized with Z-ELP for improved aggregation and mAb binding. Compared to the 50% decorated Z-ELP40-E2 nanocages produced by SrtA ligation, the fully decorated Z-ELP80-Spy-E2 nanocages exhibited a 10 °C lower transition temperature and a 2-fold higher mAb binding capacity. The improved transition property of the longer Z-ELP80 backbone allowed for >90% recovery of Z-ELP80-Spy-E2 nanocages at room temperature using 0.1 M ammonium sulfate after mAb elution. The flexibility of customizing different affinity domains onto the SpyTag-E2 scaffold should expand our ability to purify other non-mAb target proteins based on affinity precipitation.

INTRODUCTION

Affinity precipitation is a bioseparation method that involves the capture and precipitation of a target protein in bulk solution and has been proposed as a more cost-effective and scalable alternative to conventional Protein A affinity chromatography for the purification of therapeutic monoclonal antibodies (mAbs).2,3 The two main strategies for affinity precipitation utilize either multivalent affinity ligand cross-linking (primary effect) or stimuli-responsive affinity ligands (secondary effect) to trigger the formation of larger aggregates for precipitation.4 Primary effect affinity ligands such as multivalent haptenes have been used to cross-link with dimeric antibodies into oligomeric aggregates,5,6 however, this strategy is limited by low precipitation yields, slow dissolution kinetics, and the requirement of an additional separation step after elution to remove the dissociated affinity ligands.7 mAb purification using stimuli-responsive affinity ligands has also been implemented using either pH8 or temperature9 responsive synthetic polymers and biopolymers such as elastin-like polypeptides (ELP).10,11 However, utility of the technique is hindered by the harsh environmental stimuli such as low pH, high temperature, and/or high salt concentration necessary to induce phase transition of the affinity ligand, resulting in an increase in mAb aggregation12,13 or the coprecipitation of other cell culture impurities.14

Our group has developed a new protein-based affinity scaffold that combines the benefits of both primary and secondary effect affinity precipitation. We functionalized the surface of a self-assembled 60-mer E2 nanocage with an affinity ligand consisting of a Protein A derived Z-domain-40-repeat ELP (Z-ELP40) fusion and demonstrated binding-induced multivalent cross-linking as the initial capture mechanism of mAbs from cell culture, followed by a selective precipitation of regenerated nanocages after mAb elution for easy separation.15 The 25 nm E2 nanocage from Bacillus stearothermophilus was selected as an ideal scaffold due to its high structural stability (Tm > 80 °C) and the ability to genetically modify the exterior N-terminus for protein conjugation.16 Because large genetic fusions have been shown to disrupt proper E2 self-assembly, we modified a truncated E2 subunit with a small N-terminal triglycine tag to allow for post-translational, site-specific conjugation of Z-ELP40 using Staphylococcus aureus Sortase A (SrtA) ligation.17,18 Using partially functionalized Z-ELP-E2

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nanocages, an affinity precipitation process capable of high yield and efficient impurity clearance equivalent to Protein A chromatography was developed for the purification of industrial mAbs from cell culture harvests.19,20

In the current study, we sought to maximize the Z-ELP ligation density on the E2 nanocage using a longer chain-length 80-repeat ELP and to investigate the effects of Z-ELP valency on mAb-nanocage cross-linking and ELP phase transition properties (Figure 1). While SrtA ligation offers the benefits of using small recognition motifs (N-terminal GGG and C-terminal LPXTG) for biocompatibility,21 the reaction is reversible22,23 and resulted in a maximum Z-ELP-E2 density of about 50% (30 conjugated Z-ELP per 60 subunit E2 nanocage) even with a molar reactant ratio of 5:1 Z-ELP:E2. To achieve 100% ligation, an alternative method was evaluated using the SpyTag/SpyCatcher system derived from Streptococcus pyogenes fibronectin-binding protein FbaB, which forms an irreversible isopeptide bond between a small, 13 amino acid peptide (SpyTag) and its 15 kDa protein partner (SpyCatcher).24 Herein, we report the high expression and stable assembly of an N-terminally modified SpyTag-E2 and the tunable functionalization of both Z-ELP40-SpyCatcher and Z-ELP80-SpyCatcher fusions to SpyTag-E2 with close to 100% ligation density. We found that greater than 50% Z-ELP conjugation is sufficient for effective cross-linking with mAbs independent of ELP chain length; however, only fully decorated Z-ELP80 nanocages could be selectively recovered by precipitation at room temperature with minimal salt after mAb elution. We believe that the SpyTag-E2 nanocage system can be extended as a versatile scaffold for the functionalization of any target-SpyCatcher fusion proteins for a wide range of applications.

■ RESULTS AND DISCUSSION

Evaluation of ELP-Fusion Protein Ligation Density on E2 with Sortase A. Previous studies have demonstrated the successful purification of mAbs using Z-ELP40 functionalized E2 nanocages through binding-induced cross-linking.19 Our goal was to further improve the purification efficiency by maximizing the Z-ELP valency using a longer chain-length 80-repeat ELP. We expected that a higher density of the conjugated Z-domain would increase the extent of mAb-mediated cross-linking, while the longer ELP would decrease the salt concentration required for precipitation25 and improve nanocage regeneration. To compare the SrtA ligation efficiency of Z-ELP80-LPETG and Z-ELP80-LPETG to GGG-E2, reactants were mixed at 1:1 to 5:1 LPETG:GGG molar ratios, and the ligation products were purified using ELP inverse transition cycling (ITC).26 Even with five molar excess LPETG substrates, only a 50% ligation density was obtained for Z-ELP80-LPETG, while less than 10% ligation was observed for Z-ELP80-LPETG (Figure S1). This unexpectedly low efficiency was observed only with Z-ELP80-LPETG (Figure 2A). An

Figure 1. Scheme of research for the functionalization of high density longer chain-length Z-ELPs to E2 nanocages for improved antibody affinity precipitation and nanocage regeneration.

![Scheme of research for the functionalization of high density longer chain-length Z-ELPs to E2 nanocages for improved antibody affinity precipitation and nanocage regeneration.](image)

Figure 2. A) SDS-PAGE analysis of SrtA ligation reaction products using various ELP-LPETG substrates mixed at a 3:1 molar ratio with GGG-E2. B) Average ELP-E2 ligation density estimated by densitometry. Lane 1: ELP80-LPETG. Lane 2: Z-ELP80-LPETG. Lane 3: SP2-ELP80-LPETG. Lane 4: ELP80-LPETG. Lane 5: Z-ELP80-LPETG. Lane 6: SP2-ELP40-LPETG. Error bars represent 95% confidence intervals.

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Because of the reversibility of SrtA ligation and the limited ligation for Z-ELP<sub>80</sub>-LPETG, we decided to pursue an alternative conjugation strategy utilizing the SpyTag-SpyCatcher pair.24

Expression and Conjugation of SpyTag-E2 and Z-ELP-SpyCatcher Constructs. To overcome the limitations of the SrtA reaction, SpyTag/SpyCatcher conjugation was selected as an alternative because of its irreversible and rapid isopeptide bond formation.31 Although we were successful in displaying the GGG tag on the N-terminus of the E2 monomer, there are very few reports on the genetic fusion of other peptides onto E2.32 To identify the best insertion site, the 13-amino acid SpyTag was attached to different locations on the native N-terminal linker of the E2 monomer and expressed in E. coli. Various levels of soluble expression were obtained for all SpyTag-E2 fusion constructs (Figure S4A). The soluble E. coli lysate was heated to 70 °C for the partial purification of the E2 nanocages and to check for thermostability (Figure S4B). All SpyTag-E2 constructs were at least partially soluble after 70 °C incubation, and dynamic light scattering confirmed assembled nanoparticles of the expected size (Figure S5A). Z-ELP<sub>40</sub>-SpyCatcher and Z-ELP<sub>80</sub>-SpyCatcher were expressed and purified using ITC (Figure S6). SpyTag-E2(158) and SpyTag-E2(173) were selected to test for conjugation with the Z-ELP-SpyCatcher fusions because they exhibited the highest culture productivity and low polydispersity (Figures S4C and S5B). Samples were mixed for 1 h in PBS followed by one ITC cycle to remove any unreacted E2. The reaction resulted in high conjugation of both Z-ELP<sub>40</sub>- and Z-ELP<sub>80</sub>-SpyCatcher expression on either SpyTag-E2(158) or SpyTag-E2(173) (Figure 3). SpyTag-E2(158) was selected for future investigation because it contained the same N-terminal extension as the well-characterized GGG-E2(158) from our previous studies.27 for better comparison.

To compare the two conjugation methods, all reactants were mixed at a 0.75:1 ELP:E2 molar ratio for 4 h followed by one ITC cycle (Figure S7). In contrast to the low conversion of the SrtA ligation, SpyTag/SpyCatcher conjugation resulted in close to 100% efficiency for both Z-ELP<sub>40</sub>- and Z-ELP<sub>80</sub>-SpyCatcher, as no unreacted Z-ELP was detected by SDS-PAGE in the purified products. The simplicity of the 2-component reaction and the high efficiency afforded by SpyTag/SpyCatcher significantly advance the capabilities of the nanocage affinity precipitation technology and enable access to all 60 E2 subunits for a highly tunable platform.

Figure 3. Conjugation of SpyTag-E2(158) (Lane 1) and SpyTag-E2(173) (Lane 2) with Z-ELP<sub>40</sub>-SpyCatcher (Lane 3) and Z-ELP<sub>80</sub>-SpyCatcher (Lane 4) using either a 2:1 or 5:1 molar ratio of SpyCatcher:SpyTag substrates. Conjugation products were analyzed by SDS-PAGE for Z-ELP<sub>40</sub>-SpyCatcher and SpyTag-E2(158) (Lanes 5–6), Z-ELP<sub>40</sub>-SpyCatcher and SpyTag-E2(173) (Lanes 7–8), Z-ELP<sub>80</sub>-SpyCatcher and SpyTag-E2(173) (Lanes 9–10), and Z-ELP<sub>80</sub>-SpyCatcher and SpyTag-E2(173) (Lanes 11–12).

Effect of Ligation Density on Nanocage Aggregation and IgG Binding Capacity. To examine the versatility of the SpyTag/SpyCatcher system to generate a wide range of Z-ELP-functionalized E2 nanocages, reactions were prepared at 0.25:1, 0.5:1, 0.75:1, and 1:1 Z-ELP:E2 molar ratios for the 40- and 80-repeat ELP. For comparison, SrtA ligation reactions were used to prepare different levels of functionalized E2 nanocages using 0.5:1, 1:1, 2:1, and 4:1 Z-ELP<sub>80</sub>-E2 molar ratios (Figure 4A). The E2 ligation density was proportional to the reactant ratio for the SpyTag/SpyCatcher system, while a significant excess amount of Z-ELP was required to drive the conversion to 50% for the SrtA ligation (Figure 4B). The average conjugation efficiency for the SpyTag/SpyCatcher products exceeded 90% (Figure 4C), indicating that any target Z-ELP conjugation density may be achieved by simply mixing at the target Z-ELP:E2 reactant ratio. Because the SpyTag/SpyCatcher system eliminates the requirement for SrtA and the need to remove excess Z-ELP reactants, this system offers a substantially more streamlined and cost-efficient method to generate Z-ELP functionalized E2 nanocages.

The potential benefits of higher Z-ELP ligation densities on the nanocage transition temperature (T<sub>T</sub>) and IgG binding capacity were further investigated. Increasing the ligation density from 50% to 100% resulted in a 5 °C lower T<sub>T</sub>, and conjugation with the longer 80-repeat ELP further decreased the T<sub>T</sub> value by 6–7 °C across all samples (Figure 5A). These results again highlight the benefit of the SpyTag/SpyCatcher system as only the fully decorated Z-ELP<sub>80</sub>-E2 nanocage provides a T<sub>T</sub> value close to room temperature, a condition highly desirable for nanocage recovery using only a small increase in ionic strength after mAb elution.

To ensure that the longer ELP and the presence of a larger SpyCatcher fusion have no impact on IgG binding, the binding capacity per nanocage was evaluated using an industrial mAb (human IgG1) in the presence of excess IgG. As expected, a higher Z-ELP ligation density resulted in a higher binding capacity, and the ELP chain-length had no influence in mAb
binding (Figure 5B). For the ∼100% ligation samples, the expected molar interaction stoichiometry of 2:1 Z:IgG was observed with approximately 30 IgG molecules bound per 60 E2 subunits, whereas the low ligation samples (≤25% Z-ELP) bound IgG at a slightly lower ratio. The high local Z-domain concentration on the high ligation nanocages likely favors 2:1 Z:IgG binding, while at low ligation, the Z-domains may be more sterically restricted for coordinated binding to the same IgG molecule. Collectively, these results demonstrate a new conjugation platform capable of generating E2 nanocages with tunable Tt and high mAb binding capacity without limitations on maximum ligation density and ELP chain-length.

**Effect of Ligation Density on mAb-Nanocage Cross-Linking and Precipitation Yield.** To investigate whether the higher ligation density can also enhance mAb-nanocage cross-linking and mAb precipitation yield, Z-ELP<sub>40</sub>-Spy-E2 or Z-ELP<sub>80</sub>-Spy-E2 nanocages were mixed with an industrial mAb at 1:1, 2:1, and 4:1 Z:mAb molar ratios. Samples were incubated for 15 min to reach a steady state based on previous analysis of aggregation kinetics. The sample turbidity (350 nm) was used to indicate the level of nanocage cross-linking by detecting the formation of large particles based on light scattering. As with the transition temperature, the level of cross-linking increased continuously with ligation density for all molar ratios tested (Figure 6A and 6B). This result is consistent with our proposed mechanism<sup>15</sup> as higher ligation densities promote more interparticle cross-linking and the formation of larger aggregates due to the multivalency effect (Figure 7). Since similar results were obtained for the 40 and 80-repeat Z-ELPs, this suggests that ELP chain-length has no impact on the mAb-Z-domain interaction.

To examine whether higher nanocage cross-linking can also improve the mAb precipitation yield, samples were centrifuged at 23 °C to pellet only the cross-linked, insoluble aggregates. The percentage of captured mAb increased with the ligation density until 50%, beyond which only a slight change in the mAb precipitation yield was observed (Figure 6C and 6D). This result again confirms that cross-linking is the key mechanism responsible for mAb recovery; only E2 nanocages with a high valency of affinity ligands can nucleate into larger mAb-nanocage aggregates that are efficiently separated from other soluble solution components by centrifugation. Meanwhile, at lower ligation densities, the mAb-nanocage mixtures did not form sufficient cross-links for phase separation, as evidenced by the lower turbidity and mAb precipitation yields. It is interesting to note that a slight excess of Z-domain from the expected binding stoichiometry of 2:1 was required to achieve the highest mAb recovery. This may be due to the modest binding affinity between the Z-domain and IgG (∼200 nM), resulting in the need for a higher Z-domain ratio to achieve better mAb binding.

**Selective Precipitation and Regeneration of Nanocages after mAb Elution.** One drawback with the partially functionalized E2 nanocages generated by SrtA ligation is the need of 0.3 M ammonium sulfate for their selective precipitation and separation from the eluted mAbs. From an industrial perspective, it is desirable to minimize the salt concentration because exposure to high ionic strength at low
precipitation was evaluated at Z-ELP-Spy-E2 concentrations ranging from 25 to 75 μM (Figure S8). As expected, the fully decorated Z-ELP80-Spy-E2 nanocages exhibited higher precipitation yields over the entire salt concentration range. At a nanocage concentration used in a typical mAb purification process (75 μM Z-ELP-E2), 95% precipitation was achieved for Z-ELP80-Spy-E2 with 0.1 M ammonium sulfate, while 0.3 M ammonium sulfate was required to achieve the same yield for Z-ELP40-Spy-E2 (Figure 8A). Although the longer ELP backbone provides only a modest benefit in mAb capturing, it offers a significant advantage in nanocage recovery because of the superior phase transition property (Table 1). These enhanced aggregation properties also prevent leaching into the final mAb supernatant and reduce loss of nanocages during regeneration.

Overall, mAb purification using Z-ELP80-Spy-E2 and 0.1 M ammonium sulfate resulted in efficient nanocage regeneration with no detectable leaching of E2 nanocages in the eluted mAb (Figure 8B). In comparison, the fully ligated Z-ELP40-Spy-E2 nanocage provides comparable mAb yields and significantly higher nanocage recovery compared with the 50% ligated Z-ELP40-SrtA-E2 nanocage (Table S1). These improvements make the SpyTag-SpyCatcher-based affinity precipitation platform a more viable alternative to Protein A chromatography for mAb purification.

CONCLUSION

In the current work, we sought to improve the functionalization of E2 protein nanocages for mAb affinity precipitation by increasing the ligation density using longer chain-length Z-ELP affinity ligands. We expected that a 100% ligated Z-ELP-E2 nanocage with a longer ELP backbone would decrease the salt concentration required for nanocage precipitation and increase antibody binding capacity. SrtA ligation was originally used but less than 10% ligation efficiency was obtained using an 80-repeat Z-ELP. This was attributed to steric interference of the SrtA active site by the disordered nature of the longer ELP in combination with the Z-domain fusion.

To bypass this limitation, a SpyTag/SpyCatcher ligation strategy was evaluated as an alternative conjugation method. SpyTag was fused to the N-terminus of the E2 subunit, and a high soluble expression of assembled nanoparticles was obtained. The reaction of Z-ELP40-Spy-E2 fusion proteins with SpyTag-E2 resulted in close to 100% ligation efficiency, allowing any Z-ELP-E2 decoration density to be achieved by simply mixing at the target Z-ELP:E2 reactant ratio. Compared to the 50% Z-ELP-decorated nanocages obtained using SrtA, the fully decorated Z-ELP40-Spy-E2 nanocages exhibited a 10 °C lower transition temperature and a 2-fold higher IgG binding capacity. The highly decorated nanocages (greater than 50%) can efficiently cross-link with mAb into large aggregates, enabling the capture of mAbs without the use of salt. The 100% ligation Z-ELP80-Spy-E2 nanocages allowed selective nanocage precipitation and recovery after mAb elution in the presence of 3-fold less salt (0.1 M ammonium sulfate) than using the shorter Z-ELP40.

pH can result in increased aggregation for some mAbs. Because of the 5 to 10 °C lower Tt value for the fully decorated E2 nanocages, we next investigated whether this can translate into improved precipitation yields at lower salt concentrations. The salt concentration required for greater than 95% nanocage precipitation was evaluated at Z-ELP-Spy-E2 concentrations ranging from 25 to 75 μM (Figure 8B). In comparison, the fully decorated Z-ELP80-Spy-E2 nanocages exhibited higher precipitation yields over the entire salt concentration range. At a nanocage concentration used in a typical mAb purification process (75 μM Z-ELP-E2), 95% precipitation was achieved for Z-ELP80-Spy-E2 with 0.1 M ammonium sulfate, while 0.3 M ammonium sulfate was required to achieve the same yield for Z-ELP40-Spy-E2 (Figure 8A). Although the longer ELP backbone provides only a modest benefit in mAb capturing, it offers a significant advantage in nanocage recovery because of the superior phase transition property (Table 1). These enhanced aggregation properties also prevent leaching into the final mAb supernatant and reduce loss of nanocages during regeneration.

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To bypass this limitation, a SpyTag/SpyCatcher ligation strategy was evaluated as an alternative conjugation method. SpyTag was fused to the N-terminus of the E2 subunit, and a high soluble expression of assembled nanoparticles was obtained. The reaction of Z-ELP40- or Z-ELP80-SpyCatcher fusion proteins with SpyTag-E2 resulted in close to 100% ligation efficiency, allowing any Z-ELP-E2 decoration density to be achieved by simply mixing at the target Z-ELP:E2 reactant ratio. Compared to the 50% Z-ELP-decorated nanocages obtained using SrtA, the fully decorated Z-ELP40-Spy-E2 nanocages exhibited a 10 °C lower transition temperature and a 2-fold higher IgG binding capacity. The highly decorated nanocages (greater than 50%) can efficiently cross-link with mAb into large aggregates, enabling the capture of mAbs without the use of salt. The 100% ligation Z-ELP80-Spy-E2 nanocages allowed selective nanocage precipitation and recovery after mAb elution in the presence of 3-fold less salt (0.1 M ammonium sulfate) than using the shorter Z-ELP40.
Compared to SrtA-mediated ligation, the SpyTag/SpyCatcher isopeptide bond formation is fast and irreversible, enabling the economical generation of nanocages with tunable ligation densities with close to 100% conversion. Because of these benefits, we believe the SpyTag-E2 nanocage could be an ideal scaffold for the efficient, site-specific conjugation of other SpyCatcher fusion proteins. Furthermore, conventional primary effect cross-linking requires multivalent affinity ligand binding to a multimeric protein and has not been demonstrated for the capture of monomeric proteins. However, two or more affinity domain-SpyCatcher fusions with orthogonal binding sites on the same protein can be functionalized to the SpyTag-E2 scaffold that may allow for cross-linking with monomers. Future work will seek to establish a generalized affinity precipitation platform that can be implemented for the purification of any target multimeric or monomeric protein using customizable affinity ligands.

**EXPERIMENTAL PROCEDURES**

**Materials.** Escherichia coli strain BLR(DE3) containing pET24(a) vectors encoding for Z-ELP[KV₄-F-40]-LPETG, ELP[KV₄-F-40]-LPETG, ELP[KV₄-F-80]-LPETG, and GGG-ELP[KV₄-F-80]. E. coli strain BL21(DE3) containing a pET11(a) vector encoding for GGG-E2(158), and another BL21(DE3) strain containing a pMRS vector encoding for sortase A were constructed and described previously. pDEST14-SpyCatcher was a gift from Mark Howarth (Addgene plasmid # 35044). pET11(a) vectors containing SpyTag insert were ligated into the NdeI and NheI digested pE2 constructs. See Table S3 for primer/oligo information.

**Protein Expression and Purification.** All ELP-fusion constructs 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 and were purified by inverse transition cycling (ITC) using 0.5 M ammonium sulfate, as described previously. The purified ELP-fusion concentration was estimated by absorbance at 280 nm (UV-1800, Shimadzu) using the theoretical extinction coefficient. Sortase A was expressed in BL21(DE3) E. coli grown in Luria–Bertani Medium (LB) with 50 μg/mL kanamycin at 37 °C and 250 rpm until an OD600 of 1.0, where the culture was induced with 1 mM IPTG for 4 h. All E2 constructs (GGG-E2(158) and SpyTag-E2(152–179)) 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 4,000 × 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 Super Acrodisc syringe filter. E2 nanocage assembly was confirmed by dynamic light scattering (DLS) using a Malvern Zetasizer Nano (Malvern, United Kingdom) and a low volume cuvette (ZEN0040, Malvern). Each measurement consisted of 5 runs of 10 s, and the correlation function was analyzed by the Protein Analysis algorithm provided by the Malvern software to estimate the hydrodynamic diameter (Z-ave) and polydispersity index. Total protein concentration of partially purified E2 and soluble SrtA lysate was measured by Bradford.

**Genetic Manipulations and Vector Construction.** E. coli strain NEB S-alpha (NEB #C29871) was used as the host for all genetic manipulations. All ELP constructs were cloned in pET24(a) vectors, and all SpyTag-E2 constructs were cloned in pET11(a) vectors. Z-ELP₄₀-LPETG was constructed by PCR of the Z-domain from Z-ELP[KV₄-F-40]-LPETG using Primer 1 and Primer 2, and the PCR amplified Z-domain was digested with XbaI and NdeI and ligated into XbaI and NdeI digested ELP[KV₄-F-80]-LPETG. A double-repeat streptavidin binding peptide DVEAWLDERVPLVET (SP₂, (SP-(GS)₃-(SP-(GS))₃) was generated by annealing overlapping Oligos 1–4. The annealed SP₂ was digested with Ncol and NdeI and ligated into Ncol and NdeI digested Z-ELP₄₀-LPETG and Z-ELP₈₀-LPETG vectors to form SP₂-ELP₄₀-LPETG and SP₂-ELP₈₀-LPETG constructs. An (EA₆K₃) linker was created by annealing Oligos 5–6 and was substituted for the LPETG tag by digesting the Z-ELP₄₀-LPETG and Z-ELP₈₀-LPETG vectors with BamHI and SacII and ligationing the BamHI and SacII digested (EA₆K₃) linker. Z-ELP₄₀-SpyCatcher and Z-ELP₈₀-SpyCatcher were generated by PCR of the pDEST14-SpyCatcher vector using Primer 3–4, and the PCR amplified SpyCatcher was digested with BamHI and Xhol and ligated into BamHI and Xhol digested Z-ELP₄₀-LPETG and Z-ELP₈₀-LPETG vectors to replace the LPETG tag. SpyTag-E2 constructs (SpyTag-E2(152), SpyTag-E2(158), SpyTag-E2(167), SpyTag-E2(173), SpyTag-E2(179)) were generated by annealing SpyTag (AHIVMDAYKPTK) Oligos 7–8 and digesting with NdeI and NheI. The SpyTag insert was ligated into the NdeI and NheI digested pE2 constructs. See Table S3 for primer/oligo information.
proteins were purified by mass balance. The mAb binding capacity per 60-subunit E2 nanocage was calculated by dividing the mAb concentration by 60.[E2].

**mAb-Nanocage Cross-Linking and Precipitation Yield.** An industrial mAb was prepared in microcentrifuge tubes with the Z-ELP<sub>40</sub>-Spy-E2 or Z-ELP<sub>80</sub>-Spy-E2 nanocages at 1:1, 2:1, and 4:1 Z:mAb molar ratios in triplicate. The samples were mixed for 30 min at 23 °C, and the turbidity was measured using absorbance at 350 nm. The samples were then centrifuged at 15,000 × g for 10 min at 23 °C to pellet the insoluble components and the supernatant was removed. The pellet was resuspended in 1x volume elution buffer (50 mM sodium citrate, pH 3.5) and mixed for 15 min at 23 °C. The samples were adjusted to 0.5 M ammonium sulfate and centrifuged at 15,000 × g for 10 min at 23 °C to selectively precipitate the dissociated nanocage. The elution supernatant was removed, and the absorbance was measured at 280 nm to evaluate mAb precipitation yield by mass balance. The purity of the mAb elution samples was confirmed by SDS-PAGE to ensure no residual nanocage.

**Nanocage Regeneration and mAb Affinity Precipitation.** 100% ligation Z-ELP<sub>40</sub>-Spy-E2 or Z-ELP<sub>80</sub>-Spy-E2 precipitation yield in elution buffer was evaluated using a central composite DOE design (25, 50, and 75 μM E2 concentration and 0.05, 0.15, and 0.25 M ammonium sulfate) with three replicates of the centerpoint (50 μM and 0.15 M). After adding ammonium sulfate at 23 °C, the samples were centrifuged at 15,000 × g for 5 min at 23 °C, the supernatant absorbance at 280 nm was measured to evaluate nanocage concentration, and the nanocage precipitation yield was calculated by mass balance. The effect of ammonium sulfate on nanocage regeneration during mAb affinity precipitation was studied. The nanocage was mixed with an industrial mAb cell culture harvest sample at a 3:1 Z:mAb molar ratio in cell culture media in triplicate for 5 min at 23 °C and centrifuged at 15,000 × g for 5 min at 23 °C. The pelleted nanocage-complex was washed with PBS followed by 25 mM sodium citrate pH 5.0. After another centrifugation, the pellet was resuspended in elution buffer and mixed for 15 min. The elution samples were adjusted to either 0.1 or 0.3 M ammonium sulfate for selective nanocage precipitation. The purified mAb was removed in the supernatant, and mAb elution yield was calculated by measuring absorbance at 280 nm. The pelleted nanocage was regenerated in elution buffer and then buffer exchanged back into PBS using ITC. The nanocage recovery was evaluated after one purification cycle by measuring absorbance at 280 nm.

**ASSOCIATED CONTENT**

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.bioconjchem.8b00458.

Supporting figures and tables on the Sortase A ligation reactions, SpyTag-E2 expression and characterization, comparison of conjugation methods, and genetic manipulations (PDF).

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REFERENCES
