Electronic Supplementary Material (ESI) for Chemical Communications.

Supplementary Information

Sortase-mediated multifunctionalization of protein nanocages

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Figure S1. Expression and purification of ELP-LPETG fusion in *E. coli*. Cell lysates containing ELP-LPETG (1) were subjected to two cycles of thermally triggered precipitation and resolubilization and the final purified products (2) were used for the SrtA-mediated ligation.

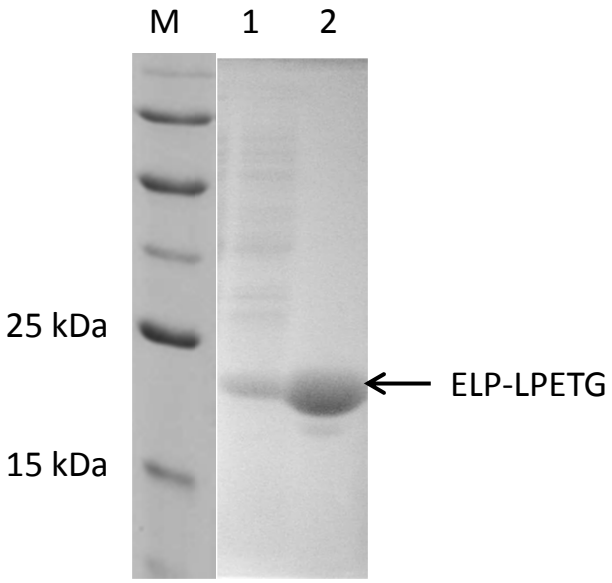


Figure S2. The transition profiles of ELP-LPETG and ELP-E2 were determined by measuring the absorbance at 650 nM using 8 µM protein and 1 M NaCl. The transition temperature was determined as the temperature where the optical density reached half of the maximum.

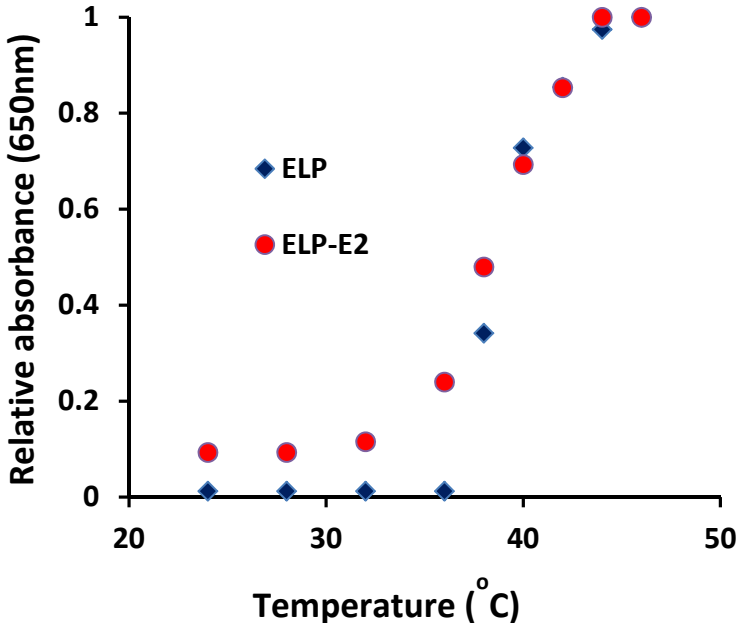


Figure S3. DLS analysis of E2, ELP-E2, and CelA/ELP-E2 conjugates. All the data were collected at 20^{o}C.

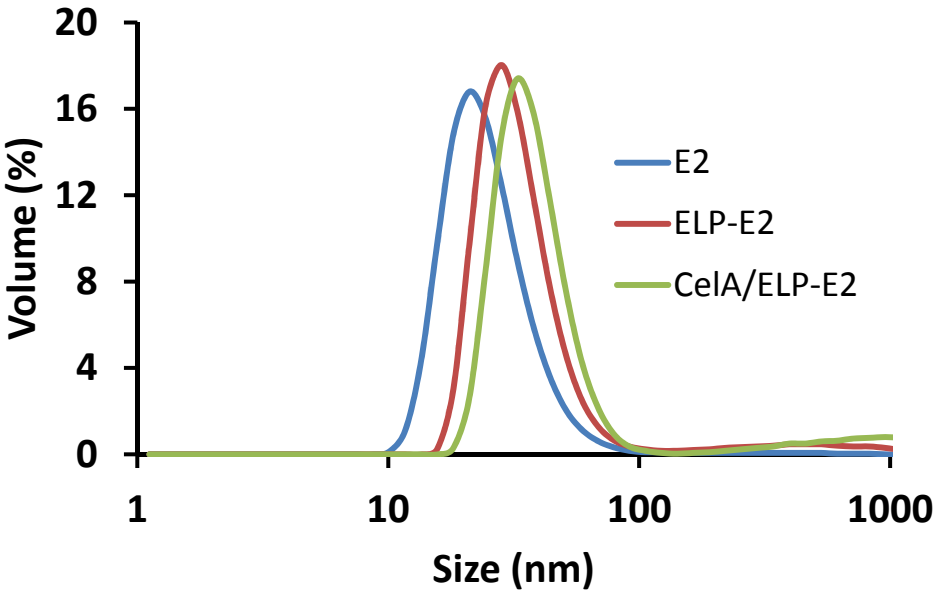


Figure S4. Expression and purification of CelA-LPETG in *E. coli*. Cell lysates containing CelA-LPTEG were heated to 50^{o}C for 10 min. The precipitates were removed by centrifugation and the remaining supernatant was the partially purified CelA-LPTEG.

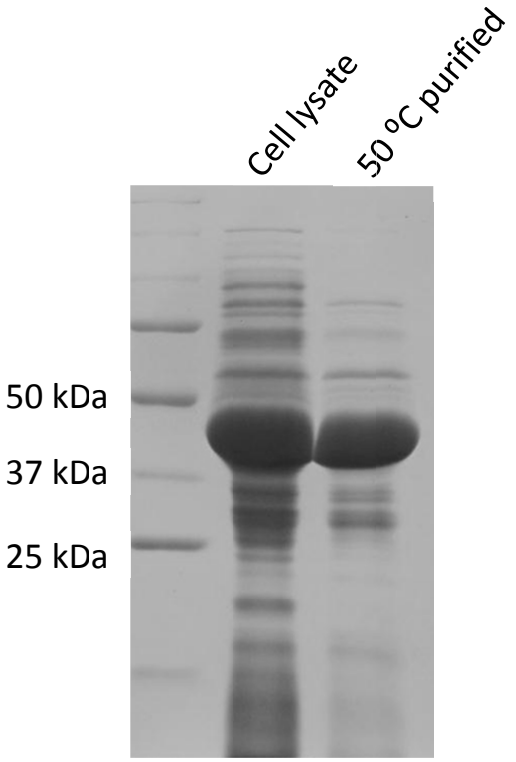


Figure S5. β-gal activity in cell lysate. Cell lysates was diluted 1500 times and the changes in OD_{420} upon the addition of o-nitrophenyl-β-D-galactoside (ONPG) is used to calculate the β-gal activity.

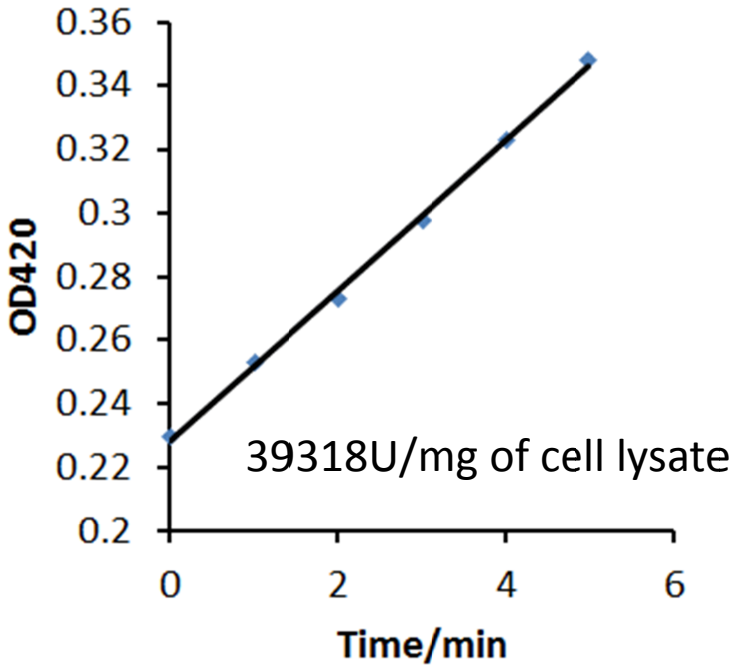
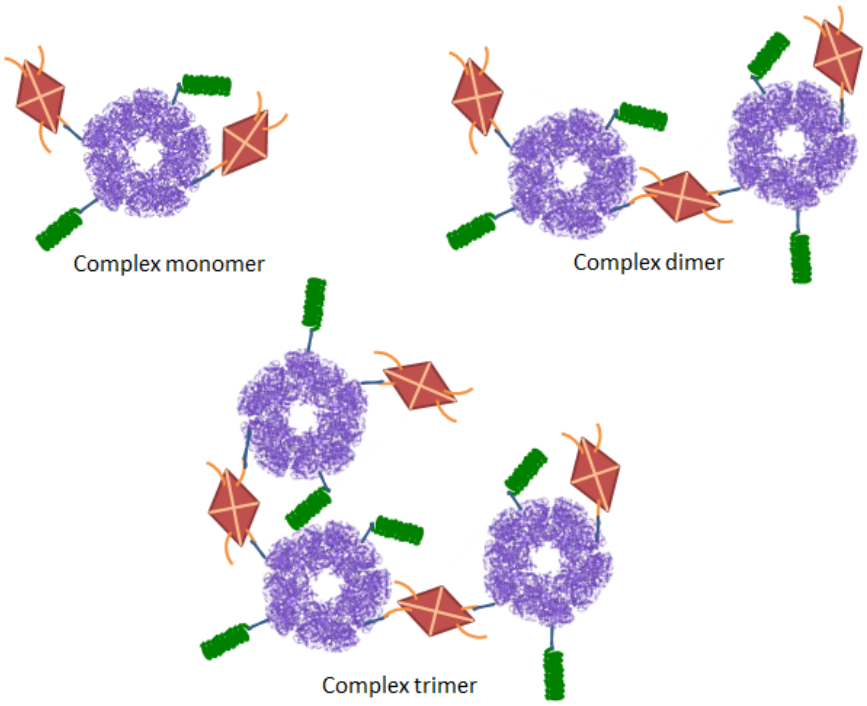


Figure S6. (A) Schematic diagram of E2 nanocage complexes forming oligomers due to ligation of E2 nanocages with the same β-gal at different β-gal monomers. (B) Analysis of β-gal/ELP-E2 conjugates by transmission electron microscopy. Most of the conjugates are individual E2 nanocage complexes (red) with a few complex dimers (blue) and complex trimmers (green).

(A)



(B)

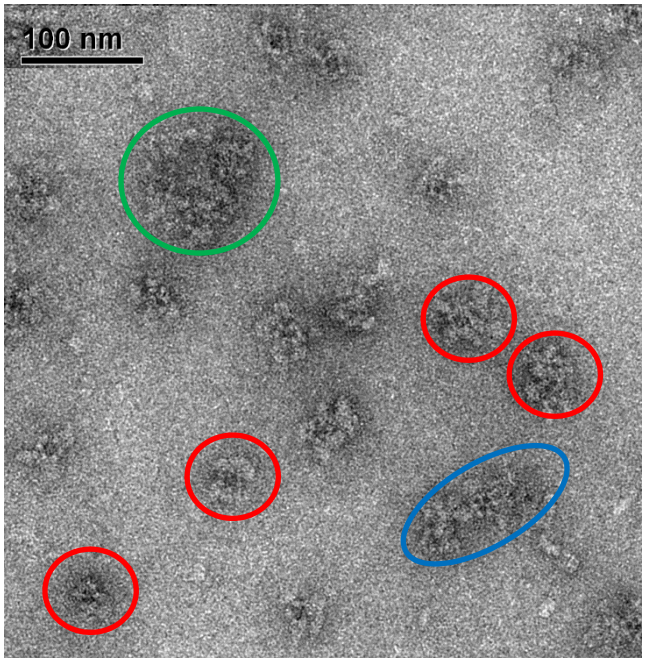
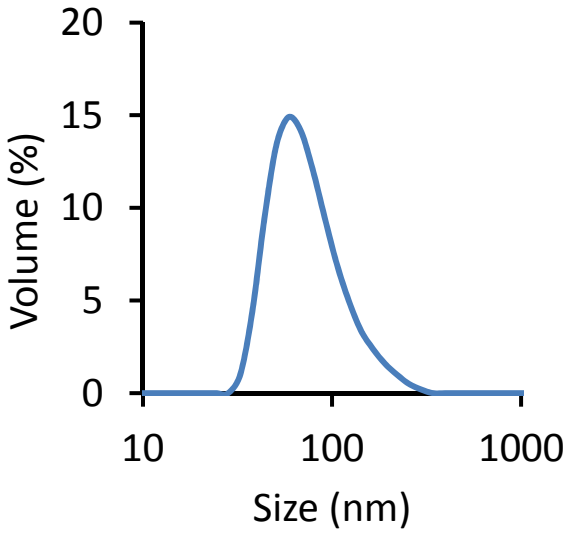


Figure S7 DLS Analysis of β-gal/ELP-E2 conjugates.



DETAILED EXPERIMENTAL SECTION

Design of plasmid with E2 gene

The E2 scaffold containing a flexible linker region for the presentation of peptides was created. The gene for this scaffold was based on a previously-published plasmid, pE2[1], which encodes for the E2 structural core protein in a pET-11a expression vector. Silent mutations were made in the E2 gene to introduce an *Xma1* restriction site at base pairs 13-18 (CCCGGG)[2]. To incorporate the native linker region, the N-terminus was extended from proline-175 to valine-158 (with amino acid site numbering corresponding to NCBI, accession number P11961). Valine-158 lies in a loop region near the N-terminus of an α-helix that extends away from the structural E2 core[2], potentially allowing for insertion of peptides with reduced steric interactions. The forward and reverse primers for creating this extended N-terminus region were 5'-GATATACATATGGCTAGCGTGCTGAAAGAAGACATTGATGCGTTTCTGGCG-3' and 5'-CCCTTTCCCGGGTTTCGCGCCGCCCGCCAGAAACGCATCAATGTC-3', respectively. Primers were heated to 90°C for 5 min, slowly cooled to 4°C to allow annealing, extended at 37°C for 2 hours with 20 U/mL T4 polymerase (New England Biolabs) and dNTP (0.5 mM each nucleotide; New England Biolabs), and brought to 75°C for enzyme inactivation. This product was then digested with *Nde1* and *Xma1* and ligated into the corresponding endonuclease sites of the pE2 plasmid [E2(158)]. The expression and solubility of this resulting protein was confirmed.

Construction of expression vectors

His6-Sortase A was expressed from pMR5. GGG-E2(158) was constructed by amplifying E2(158) [E2 encoding amino acids 158-427] fragment with the forward primer 5'-GCGCCATATGGGCGGTGGAGCTAGCGTGCTGAAAGAAGAC-3' and the reverse primer 5'-GCGCGGATCCTTAAGCTTCCATCAGC-3' and the PCR product was then digested and ligated into *Nde*I and *Bam*HI digested pET11(a). ELP[KV_8F-40]-(G4S)3-LPETG-His6 was constructed by overlapping oligonucleotides of the (G4S)3-LPETG-His6 fragment with *Bam*HI and *Xho*I overhangs and ligated with *Bam*HI and *Xho*I digested pET24(a)-ELP[KV_8F-40] to form pET24(a)-ELP[KV_8F-40]-(G4S)3-LPETG-His6. To construct the expression vector of CelA-(G4S)3-LPETG-His6, the CelA fragment was amplified with the forward primer 5'-GCGCGCTAGCATGGCAGGTGTGCCTTTTAACACAAAATAC-3' and the reverse primer 5'- GCGC GAGCTCGTTTCCTGTTATGTACAACAAAGTGAGC-3' and the PCR product was digested and ligated into *Nhe*I and *Sac*I digested pET24(a). The fragment of (G4S)3-LPETG-His6 was produced by overlapping oligonucleotides with *Sac*I and *Xho*I overhangs and ligated into *Sac*I and *Xho*I digested pET24(a)-CelA to form pET24(a)-CelA-(G4S)3-LPETG-His6. β-galactosidase was amplified from the chromosomal DNA of *E. coli* using the forward primer 5'-GCGCGCTAGCATGACCATGATTACGGAT-3' and the reverse primer 5'-GCGCGGATCCTTTTTGACACCAGACCAA-3'. β-galactosidase-(G4S)3-LPETG-his6 was constructed by inserting β-galactosidase in pET24(a) usng *Nhe*I and *BamH*I sites and the fragment of (G4S)3-LPETG-His6 was inserted using *Sac*I and *Xho*I sites.

Protein expression and purification.

Sortase A, CelA , ELP, E2, and β-galactosidase were expressed in BL21(DE3) *E. coli* in Luria-Bertani medium. All cell cultures were started with incubating at 37 ^{o}C. Sortase A was induced for expression when OD_{600} reaches 1 by addition of 1 mM isopropyl thioglucoside (IPTG) and grown at 37 ^{o}C for 4 h. CelA was induced when OD_{600} reaches 1 by addition of 0.2 mM IPTG and grown at 20 ^{o}C overnight. E2 cell culture was induced with 0.2 mM IPTG when reaching OD_{600} of 0.5 and grown at 20 ^{o}C overnight. β-galactosidase was induced for expression when OD_{600} reaches 0.75 by addition of 1 mM IPTG. ELP was expressed in BLR *E. coli* in Terrific Broth medium. The cell culture was grown at 37 ^{o}C until reaching OD_{600} of 1 and then moved to 25 ^{o}C overnight for leaky expression. Cells were harvested by centrifugation and resuspended in buffer of 50 mM Tris and 150 mM NaCl at pH8. Cells were lysed by sonication and the cell debris was removed by centrifugation at 16.1 k rcf for 10 min at 4 ^{o}C.

CelA and E2 were purified based on their thermal-stable property. CelA was incubated at 60 ^{o}C for 10 min and E2 was incubated at 70 ^{o}C for 10 min to denature *E. coli* proteins. The aggregates were removed by centrifugation at 16.1 k rcf at 4 ^{o}C for 10 min. ELP was purified by inverse phase transition with addition of 1M NaCl[3]. Samples were incubated at 37 ^{o}C for 10 min and the precipitates were pelleted by centrifugation at 16.1k rcf at 37^{o}C for 10 min. The pellet was solubilize in ice cold buffer and centrifuged at 16.1 k rcf at 4 ^{o}C for 10 min to remove insoluble proteins. The precipitation and resolubilization were repeated twice and the supernatant that contained ELP was collected. ELP concentration was determined by spectrophotometric measurement at 215 nm (UV-1800, Shimadzu).

Sortase A-mediated functionalization of E2 nanocages

To conjugate ELP onto E2 to get 10% (on average) of E2 monomers of one E2 cage being ligated with ELP, 60 μM of E2, 12 μM of ELP and 12 μM of Sortase A were used in a reaction volume of 400 μl. To conjugate CelA onto ELP-E2 (10% E2 monomers ligated with ELP), 1 μM of ELP-E2 cage, 24 μM of CelA and 24 μM of Sortase A were used in a reaction volume of 300 μl. Reaction mixtures were incubated at 37 ^{o}C for 4 h after addition of 10x reaction buffer (50 mM Tris, 150 mM NaCl, 60 mM CaCl2, pH8). The ligated products (CelA/ELP-E2) were collected by inverse phase transition with addition of 1 M Na_2SO_4. The precipitation and resolubilization procedures were the same as mentioned above. To conjugate β-galactosidase onto ELP-E2 (10% E2 monomers ligated with ELP), 0.28 μM of ELP-E2 cage, 34 μM of β-galactosidase and 15 μM of Sortase A were used in a reaction volume of 400 μl. The ligated products (β-galactosidase/ELP-E2) were collected by inverse phase transition with addition of 1 M NaCl and 50 μM ELP. The molar concentration of Sortase A, E2, CelA and ELP-E2 cage were estimated by densitometry analysis of SDS-PAGE. The molar concentration of ELP was calculated according to measurement at 215 nm.

Characterization of protein complex.

The ligation products were analyzed by SDS-PAGE. The ratio of ligated products onto E2 was estimated by densitometry. The hydrodynamic diameters of the complexes were measured by dynamic light scattering using Zetasizer Nano ZS (Malvern Instruments). The samples were in buffer of 50 mM Tris and 150 mM NaCl at pH8 for dynamic light scattering. Full complex assemblies were visualized by transmission electron microscopy. Samples were prepared in DDI

water and stained with 2% uranyl acetate on carbon-coated copper grids (Electron Microscopy Science). Zeiss Libra 120 Transmission Electron Microscope was used to visualize the samples with voltage of 120 kV.

Enzyme activity assay.

Carboxymethyl cellulose was used as substrate for CelA. Enzyme was assayed with 10%(wt/vol) concentration of cellulose in buffer of 50 mM Tris-HCl, 100 mM NaCl, and 10 mM $CaCl_2$ at pH6. Samples were collected periodically and mixed with equal-volume DNS reagent (10 g/l dinitrosalicylic acid, 10 g/l sodium hydroxide, 2 g/l phenol, 0.5 g/l sodium sulfite) to determine the amount of reducing sugars produced. The sample and DNS reagent mixture was incubated at 95 ^{o}C for 10 min at the absorbance at 575 nm was measured using microplate reader (Synergy H4, BioTek). For enzyme activity measured at different temperatures, substrate and buffer were preheated to that temperature before mixing with enzymes.

The activity of β-galactosidase was assayed as described. 30 µl of diluted cell lysate (diluted by 1500 times) was mixed with 3 µl of 100X Mg^{2+} solution (0.1 M $MgCl_2$ and 4.5 M β-mercaptoethanol), 66 µl of 1X ONPG (4 mg of Ortho-nitrophenyl-β-D-galactopyranoside in 1 ml of 0.1 M sodium phosphate at pH 7.5), and 201 µl of 0.1M sodium phosphate at pH 7.5. The mixture was incubated at 37 ^{o}C water bath for 5 min and the increase in absorbacne at 420 nm was measured using microplate reader (Synergy H4, BioTek) for 5 min at 37 ^{o}C.

References

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