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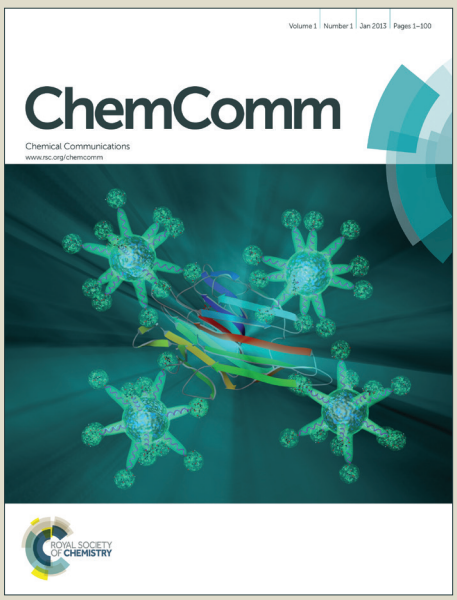

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Sortase A-mediated multi-functionalization of protein nanoparticles

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We report here a new strategy to enable fast, covalent, and site-directed functionalization of protein nanoparticles using Sortase A-mediated ligation using functional proteins ranging from monomeric to large tetrameric structures. Easy purification of the modified E2 nanoparticles is achieved by functionalization with a thermo-responsive elastin-like-peptide. The resulting protein nanoparticles remained intact and active even after repeated phase transitions, suggesting their use in biocatalysis, biosensing, and imaging applications.

Protein nanocages, such as virus capsids, have emerged as excellent building blocks for preparation of biofunctionalized nanomaterials for therapeutic diagnosis,[1,2] drug delivery,[3] and enzyme organization [4,5] because of their ability to self-assemble from simple protein building blocks.[6] These naturally derived molecular self-assembly systems not only allows precise control over material architectures and length scales, but also enables synthetic functionalization of specific regions with proteins or enzymes.[6,7] Although a wide range of proteins have been chemically conjugated onto protein nanocages using surface-exposed functional groups,[8] this method is limited because of the loss of native protein activities and the incomplete control over protein orientation.[9,10] Direct protein fusion has also been met with limited success because only short peptides or smaller proteins are usually tolerated before the ability of nanocages to self-assemble is compromised.[11,12] Click-chemistry based techniques can provide site-specific functionalization but are costly as they require incorporation of unnatural amino acids and can sometimes lower the overall protein production yield.[13] As a result, there remains an urgent need for a simple method that enables the site-specific and covalent functionalization of protein nanocages.

One attractive method is based on the *Staphylococcus aureus* Sortase A (SrtA), which catalyzes the condensation reaction between a C-terminal LPXTG motif and an N-terminal poly-glycine tag, resulting in the formation of an amide bond in a site-specific manner. [14–16] This "bio-click" chemistry approach possesses many of the same desirable features as click chemistry and can be performed under mild pH and temperature conditions.[17] Because of these favorable properties, SrtA ligation has been exploited for site-specific labeling of proteins onto solid supports,[18–20] vesicles,[21] and even capsid proteins of living M13 phages.[22]

The E2 core of the pyruvate dehydrogenase multienzyme complex from *Bacillus stearothermophilus* is a naturally occurring protein nanocage of roughly 24 nm diameter.[23,24] This E2 nanoparticle is composed of 60 identical monomers that self-assemble into a highly stable cage-like structure, and has been used as a flexible scaffold for drug conjugation and peptide display.[3,25–27] Both native and N-terminally truncated E2 subunits can be readily expressed in *E. coli,* and they maintain their stable nanocage structures even at 85°C.[28] Unfortunately, genetic fusion of proteins such as GFP to the exposed N-terminus of E2 results in only inclusion bodies, and refolding is needed to recover the intact structure.[12] Since the required peptide motifs for SrtA-ligation are small, we reasoned that multiple proteins can be easily conjugated onto E2 nanocages using this "bio-click" chemistry approach while still retaining the native protein structures.[12,17] In this paper, we successfully generated smart biocatalytic protein nanocages by simultaneously incorporating an elastin-like-peptide (ELP) as the thermo-responsive modality,[29] and either a monomeric endogluconase (CelA)[30] or a tetrameric β-galactosidase[31] as the biocatalytic component (Fig. 1).

The native E2 subunit contains a structural core domain (residue 188-427) preceded by a linker attaching two flexible domains (residue 1-187) that are used for interaction with other enzyme subunits.[12] Since deletion of the flexible domains has been shown to have no impact on the particle assembly,[28] a triglycine tag was added to the N-terminus of a truncated E2 subunit (residue 158-427) to provide sufficient spacing for protein ligation away from the E2 surface. Addition of a small triglycine tag to E2 (GGG-E2) did not affect its soluble expression in *E. coli* as a protein band corresponding to the expected size was detected on SDS-PAGE (Fig. 2A). By exploiting the thermophilic nature of E2, most cellular

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proteins were removed by denaturation simply by heating the cell lysates at 70°C for 10 min. The remaining E2 proteins maintained the nanocage structure even after this process (Fig. 2B).

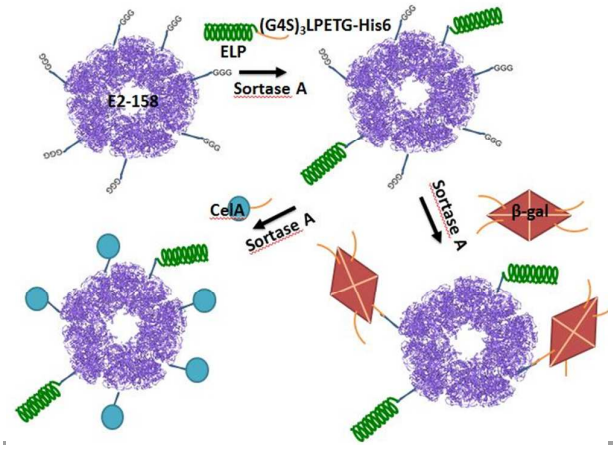

Fig.1 Schematic of the SrtA-mediated functionalization of E2 nanocages. A triglycine tag is added to the N-terminus of each E2 monomer, resulting in E2 nanocages composed of 60 GGG tags. Multiple functionalities are incorporated by sequential addition of different proteins containing a C-terminus LPTEG tag based on SrtA-mediated ligation.

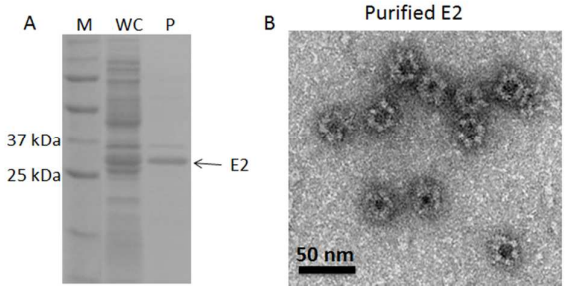

Fig.2 Production and purification of GGG-E2. (A) SDS-PAGE analysis of purification. The majority of cellular proteins were removed by heating the whole cell lysates (WC) at 70°C for 10 min followed by centrifugation, yielding purified E2 proteins (P). (B) Transmission electron micrograph of GGG-E2 confirms monodisperse, intact nanoparticles even after heat purification.

To investigate the accessibility of the N-terminus triglycine tags, we first used a thermo-responsive ELP biopolymer, ELP[KV_8F]$_{40}$, for the SrtA-mediated functionalization of E2 nanocages.[29,32,33] ELPs form hydrophobic aggregates triggered by changes in temperature and ionic strength, and have been used for the purification of a wide range of fusion partners.[34,35] We expect that partial decoration with ELP can preserve the reversible temperature-dependent aggregation, enabling easy purification of the resulting E2 nanoparticles without the use of tedious chromatography and gradient separations (Fig. 3A).[36,37] To ligate ELP to E2, an LPETG motif was added to the C-terminus of the ELP polymer; the resulting ELP-LPETG fusion proteins were purified by two cycles of precipitation/solubilization by taking advantage of the thermally induced phase transition property (Fig. S1). Purified ELP-LPETG was ligated onto E2 nanocages using a molar ratio of ELP to E2 of 1:5. This lower molar ratio was chosen to limit the level of ELP conjugation in order to allow further modifications of the E2 backbone with enzymes. As expected, only 10% of the E2 monomers were modified with ELP under this condition (approximately 6 ELPs were covalently attached per E2 nanoparticle) (Fig. 3B). The resulting ELP-E2 conjugates retained the thermo-responsive nature of ELP and intact E2 nanoparticles were easily purified after 2 cycles of precipitation and solubilization (Fig. 3B and 3C).

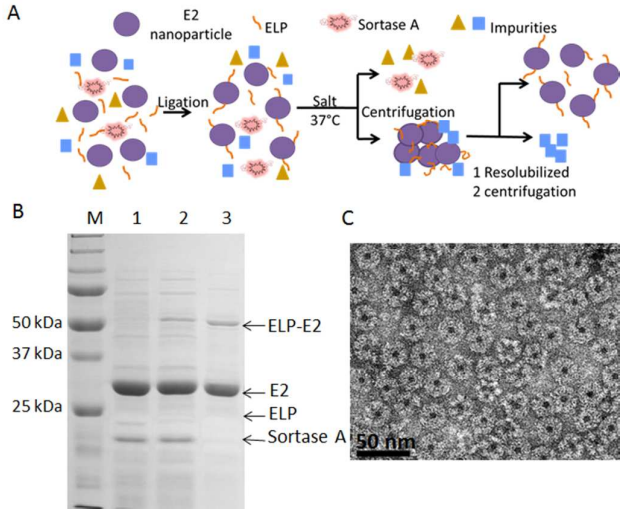

Fig.3 Preparation of ELP-E2 conjugates by SrtA-mediated ligation. (A) Conjugation and purification scheme. (B) SDS-PAGE analysis of ELP-E2 conjugation at 0 h (1) and 4 h (2). Purification of ELP-E2 conjugates by two cycles of precipitation and solubilization (3). (C) Transmission electron micrograph of 8X concentrated ELP-E2 conjugates confirms monodisperse, intact nanoparticles.

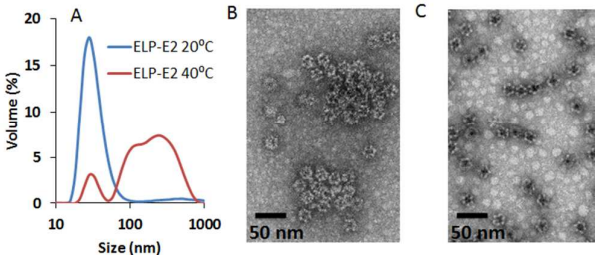

Fig.4 (A) DLS analysis of ELP-E2 conjugates at 20°C and 40°C. Transmission electron micrograph purified ELP-E2 conjugates at (B) 40°C and (C) 20°C.

The thermo-responsive properties of the ELP-E2 conjugate were further investigated by UV-VIS turbidity measurements. ELP conjugation onto E2 has a minimal impact on the transition temperature (Tt) as both ELP and ELP-E2 had a Tt value around 38°C (Fig. S2). In contrast, the E2 nanocage by itself exhibited no observable aggregation up to 60°C (data not shown), again highlighting the importance of ELP for inducing the phase transition.

Dynamic light scattering (DLS) and transmission electron microscopy (TEM) analyses were performed to further characterize the ELP-E2 conjugates. At 20°C, only intact ELP-E2 nanocages closely resembling the unmodified E2 were detected under TEM with no observable aggregation (Fig. 3C). However, DLS analysis revealed the presence of larger ELP-E2 nanoparticles (30.9 ± 1.1 nm)

compared to unmodified E2 (25.5 ± 1.2 nm), consistent with coating by a layer of ELP on the external surface (Fig. S3). Upon heating to 40°C, ELP-E2 quickly associated into larger aggregates of intact particles (Fig. 4A, 4B), while no change was observed for unmodified E2. This was confirmed by TEM and DLS, which show aggregates with sizes larger than 200 nm (Fig. 4B). More importantly, the observed aggregates disappeared upon cooling and only individual ELP-E2 nanocages were detected under TEM (Fig. 4C). Taken together, our results confirmed that conjugation of ELP to E2 allows reversible aggregation without any disruption to the E2 structure.

One key benefit of the SrtA-mediated ligation approach is the ease of multi-functionalization. To demonstrate this feasibility, a thermophilic endoglucanase, CelA, from *Clostridium thermocellum* was attached to endow the resulting nanocages with the ability to hydrolyze cellulose. An LPETG tag was added to the C-terminus of CelA, and the resulting fusion protein was successfully expressed in *E. coli* (Fig. S4). The majority of *E. coli* proteins were removed by heating the cell lysates at 50°C (Fig. S4), and the partially purified CelA was ligated to ELP-E2 by incubating with SrtA for 4 h. Products retained the ELP functionality and were recovered by two cycles of precipitation and solubilization (Fig. 5A). Successful ligation was demonstrated by three bands corresponding to CelA-E2, ELP-E2, and E2 on an SDS-PAGE gel (Fig. 5A). From band intensities, approximately 20 CelA were attached onto each E2 nanocage. Presence of intact nanocages was further confirmed by TEM, indicating that conjugation of a folded protein has no impact on the E2 structure (Fig. 5B and 5C). An external layer of CelA was visible for the CelA/ELP-E2 conjugates, yielding particles larger than ELP-E2 conjugates with an average diameter of 33.0 ± 1.6 nm by DLS (Fig. S3). Considering the size of CelA is roughly 4.5 nm,[38] the particle size is consistent with a monolayer of CelA decorating the E2 nanocage.

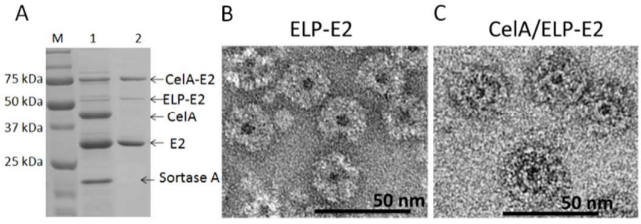

Fig. 5 Preparation of CelA/ELP-E2 conjugates. (A) SDS-PAGE analysis of CelA/ELP-E2 before (1) and after (2) purification by two cycles of precipitation and solubilization. Comparison of (B) ELP-E2 and (C) Cel/ELP-E2 conjugates by transmission electron microscopy.

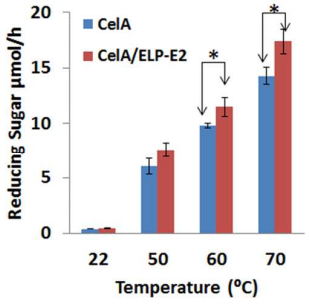

Fig.6 Functionality of CelA/ELP-E2 conjugates. CelA activity was evaluted using CMC and the resulting reducing sugar production was measured for both CelA and CelA/ELP-E2. p values <0.05 (n=3).

To examine whether the conjugated CelA retains enzymatic activity, CelA/ELP-E2 conjugates were tested for cellulose hydrolysis. Carboxymethyl cellulose (CMC) was used as a substrate, and the rates of reducing sugar production of CelA bound to E2 were compared with rates by the same amount of free enzymes at different temperatures (Fig. 6). The reaction rates of CelA/ELP-E2 increased with temperature in a similar manner as CelA, confirming that the CelA functionality was not compromised during the ligation and purification process.[39]

Although smaller monomeric proteins (e.g. GFP) have been genetically tethered onto the surface of protein nanoparticles, attempts to display larger dimeric or tetrameric proteins have failed because the resulting quaternary structures interfere with capsid particle formation.[40] To investigate whether our "bio-click" chemistry approach can be used to bypass this limitation, a tetrameric β-galactosidase (β-gal) (>110 kDa per monomer) was used to demonstrate this feasibility. Expression of the full-length β-gal-LPETG fusion was demonstrated by SDS-PAGE (Fig. 7A) and the functionality was confirmed using the Miller assay (Fig. S5). Using a ratio of E2 to β-gal of 8, successfully Srt-A mediated ligation was demonstrated by the formation of a large 145 kDa band (Fig. 7A). The resulting β-gal/ELP-E2 conjugates were further purified by using two cycles of precipitation and solubilization (Fig. 7A). In most cases, only one of the four β-gal monomers was ligated onto E2 (Fig. 7B and Fig. S6) because of the very low β-gal to E2 ratio. The un-ligated monomers on each β-gal were also co-precipitated with the E2 conjugates because of the tetrameric nature of β-gal. However, a few dimeric and trimeric β-gal/ELP-E2 conjugates were detected, indicating the cross-linking of a single β-gal tetramer with more than one E2 nanocage (Fig. 7B and Fig. S6). This was further supported by detecting particles of roughly 60 nm in size, consistent with a monolayer of tetrameric β-gal on the E2 surface (Fig. S7).[41] The functionality of the β-gal/ELP-E2 conjugates was further confirmed by activity assays (Fig. 7C). In contrast, when β-gal and ELP-E2 were incubated without sortase A, no activity was detected after ELP purification. Taken together, our results confirmed the successful ligation of larger, multimeric enzymes using this "bio-click" chemistry approach.

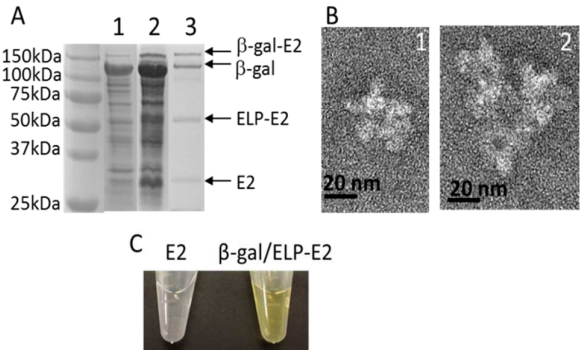

Fig.7 Preparation of β-gal/ELP-E2 conjugates. (A) Conjugation and purification of β-gal/ELP-E2 conjugates. SDS-PAGE analysis of (1) soluble cell lysates expressing the β-gal-LPETG fusion, β-gal/ELP-E2 conjugates before (2) and after (3) purification(B) Transmission electron micrograph of 2% uranyl acetate stained purified β-gal/ELP-E2 conjugates confirms the presence of (1) monomeric and (2) trimeric E2 nanoparticles. (C) Detection of β-gal activity by the release of o-nitrophenol which has a distinct yellow color.

In summary, we have developed a simple "bio-click" chemistry approach for the stepwise display of functional proteins onto E2 nanocages using genetically encoded substrates. Sequential modifications with ELP and enzymes resulted in biocatalytic and thermally responsive E2 nanocages, in which all protein components are structurally intact. Partial decoration with ELP enables easy purification of modified E2 nanoparticles by taking advantage of the reversible phase transition property of the ligated ELP backbone. Due to the simplicity of the approach, we expect this SrtA mediated "bio-click" chemistry technique to find a range of applications in functionalizing protein nanoparticles for use in biocatalysis, drug delivery, and diagnostics.

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