ChemBioChem

Research Article
doi.org/10.1002/cbic.202200332

Chemistry Europe
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Post-Assembly Modification of Protein Cages by Ubc9-Mediated Lysine Acylation

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Although viruses have been successfully repurposed as vaccines, antibiotics, and anticancer therapeutics, they also raise concerns regarding genome integration and immunogenicity. Virus-like particles and non-viral protein cages represent a potentially safer alternative but often lack desired functionality. Here, we investigated the utility of a new enzymatic bioconjugation method, called lysine acylation using conjugating enzymes (LACE), to chemoenzymatically modify protein cages. We equipped two structurally distinct protein capsules with a LACE-reactive peptide tag and demonstrated their modification with diverse ligands. This modular approach combines the advantages of chemical conjugation and genetic fusion and allows for site-specific modification with recombinant proteins as well as synthetic peptides with facile control of the extent of labeling. This strategy has the potential to fine-tune protein containers of different shape and size by providing them with new properties that go beyond their biologically native functions.

Nature has evolved an astonishing array of protein cages with unique structures and functions. The most common and well understood are viruses, which have been adapted for applications ranging from medicine to nanotechnology.[1] However, potential virulence, genome integration, and immunogenicity are serious concerns that limit their development for many medical applications. Virus-derived particles lacking a genome and non-viral protein cages represent promising alternatives due to their inherent engineerability and inability to replicate.[2] Both the interior and exterior surface of these protein shells can be modified to tune cargo preferences and interactions with the environment while retaining well-defined assembly states.[3]

For delivery applications, targeting protein cages to specific tissues, increasing their physiological half-life, and reducing immunogenicity pose substantial challenges that can be addressed by modification of the exterior surface of the particles. Three main strategies exist to functionalize protein cages: (i) genetic fusion;[4] (ii) chemical modification of natural[5] and noncanonical amino acids;[6] and (iii) chemoenzymatic methods. All are useful, but chemoenzymatic strategies are particularly attractive for cage modification because they often exhibit high site- and/or sequence-specificity, resulting in well-defined conjugates. A variety of enzymes are available for derivatization of protein cages, including sortase,[7] tyrosinase,[8] asparaginyl endopeptidase,[9] glycosyltransferase,[10] and transglutaminase.[11] Although they catalyze different reactions, these enzymes all operate under mild reaction conditions and require only canonical amino acids.

In this report, we document the utility of lysine acylation using conjugating enzymes (LACE)[12] for post-assembly decoration of non-viral protein compartments (Figure 1). LACE relies on the SUMO-conjugating enzyme Ubc9 to conjugate peptide thioesters that contain a C-terminal ubiquitin-derived sequence (LRLRGG) to a lysine residue located in a recognition sequence (IKQE) via an isopeptide bond. This chemoenzymatic approach is an attractive choice for cage modification because: (i) the genetically encoded consensus sequence is one of the shortest known for enzymatic protein modification;[13] (ii) targeting of the lysine side chain makes conjugation at internal sites and protein loops possible; (iii) Ubc9 is compatible with a range of thioester substrates, from small molecules to proteinaceous ligands; and (vi) the reaction is irreversible.

As representative protein cages, we chose variants of *Aquifex aeolicus* lumazine synthase (AaLS) and the computation-

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Supporting information for this article is available on the WWW under https://doi.org/10.1002/cbic.202200332

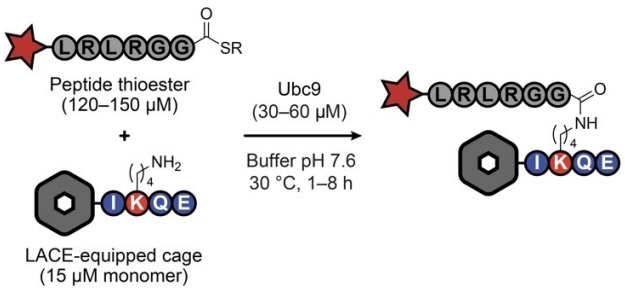

Figure 1. General strategy for post-assembly modification of protein cages by LACE.

ally designed cage O3-33.[14] These two structurally diverse protein cages have proven highly effective at encapsulating a wide range of cargo molecules via electrostatic interactions.[15] AaLS, for example, has been engineered and evolved[16] to bind positively charged cargo. One variant, AaLS-13[17] (Figure 2a), is a large (38 nm), 360-subunit cage with a negatively charged lumenal surface that allows encapsulation of positively charged proteins at rates approaching the diffusion limit.[18] Similarly, the computationally designed O3-33 cage, a smaller (14 nm), 24-subunit assembly, served as a starting scaffold for rational engineering of a porous capsule with a positively charged interior.[19] The resulting variant, OP (Figure 2b), efficiently encapsulates oligonucleotides *in vitro* and can deliver them to the cytoplasm of mammalian cells for gene knockdown.[19] Furthermore, incorporation of anionic lipids into the OP lumen gives rise to hybrid assemblies with a micellar core that encapsulate and deliver small molecules.[20]

Our main consideration when designing protein cage variants equipped with a LACE tag was accessibility of the

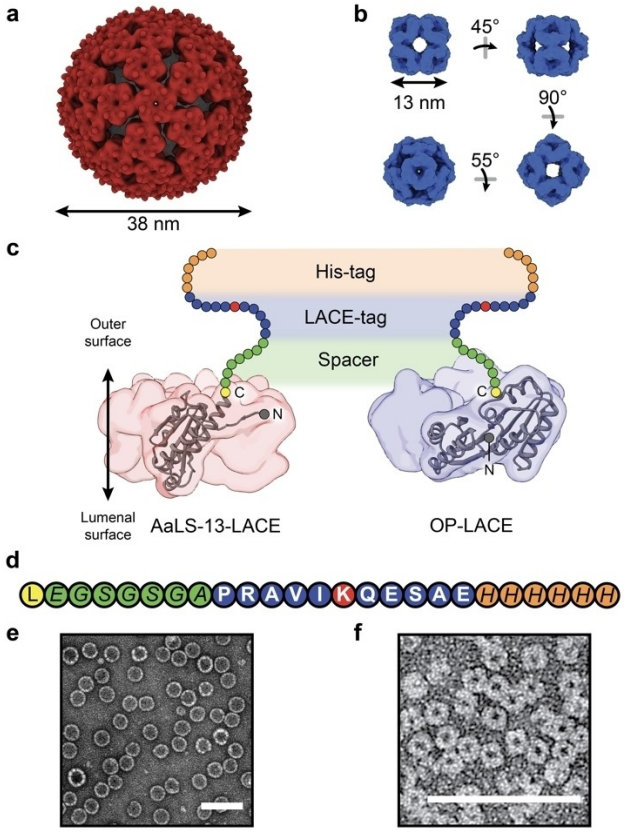

Figure 2. Design of AaLS-13 and OP cages for modification by LACE. Surface representations of (a) AaLS-13 (PDB 5MQ7) and (b) OP (PDB 6FDB) cages. (c) Transparent surface of a pentamer used to construct an AaLS-13 cage (left) and a trimer used to construct OP (right). Monomers are shown as gray ribbons. The N-terminus is highlighted as a gray sphere (Met1 for AaLS-13 and Ser2 for OP). The C-terminus is highlighted as a yellow sphere (Leu155 for AaLS-13 and Leu185 for OP). The LACE tag (blue spheres with the lysine residue highlighted in red) was linked to the surface exposed C-termini of both constructs via a flexible linker (green spheres). The hexahistidine tag used for purification is represented as orange spheres. (d) Amino acid sequence of the tag. TEM images of (e) AaLS-13-LACE and (f) OP-LACE. Scale bar is equal to 100 nm.

recognition motif (IKQE) by Ubc9. The icosahedral AaLS-13 is composed of 72 pentameric capsomers, with intra- and inter-capsomer distances between the C-termini of approximately 31 Å (Figure S1a). The C-terminus of AaLS-13 has been utilized to display protein ligands for further functionalization with antibodies[21] and enzymes.[9–10] In contrast, OP consists of eight trimeric capsomers with octahedral symmetry, in which the intra-capsomer distance of approximately 17 Å between the C-termini is much smaller than the inter-capsomer distance (53 Å) (Figure S1b). To ensure accessibility to Ubc9, we genetically fused the recognition peptide to the C-terminus of both protein cages as part of a flexible LACE tag (PRAVIKQESAE) embedded between a seven-amino acid long spacer and a hexahistidine tag (Figures 2c,d and S1, Table S1). The resulting constructs were successfully expressed in *Escherichia coli* in similar yield as their parent proteins. Analysis of the purified proteins by size-exclusion chromatography (SEC) (Figure S2) and transmission electron microscopy (TEM) (Figure 2e,f) showed that the LACE-equipped variants adopted the same core structures as their parent cages. Mass spectrometry (MS) confirmed that the LACE tag was present on every monomer (Figure S3).

We first modified LACE-equipped AaLS cages with peptide thioesters bearing rhodamine (**1**) and biotin (**2**) (Figure 3a). Modification of cages with these small molecule probes can be used for fluorescent imaging and as affinity handles, respectively. We first reacted AaLS-13-LACE (15 μM monomer) with rhodamine thioester **1** (120 μM) for 1 h in the presence of Ubc9 (30 μM) (Table S2, Entry 1). Over the course of the reaction, Ubc9 also underwent self-labeling with the peptide thioester (Figure 3b). Unreacted substrates, Ubc9 and self-labeled Ubc9 were separated from the cage products by SEC (Figure S4a) to give AaLS-13-LACE cages with approximately 10% of the subunits labeled with rhodamine as judged by SDS-PAGE (Figure 3b). TEM confirmed that the rhodamine-labeled AaLS-13-LACE cages remained intact (Figure S4b). Next, we attempted to increase the extent of cage modification by increasing the amount of Ubc9 (60 μM) and thioester **1** (150 μM) (standard conditions), as well as extending the reaction time. However, precipitation of the labeled cage was observed within 2 h, which was likely caused by over-labeling of the AaLS-13-LACE cage with the hydrophobic rhodamine dye.

Using the biotin thioester **2** substrate, higher degrees of cage labeling could be reached compared to rhodamine. The labeling extent could be tuned from 20% up to 90% by lengthening the reaction time from 2 h to 8 h (Table S2, Entries 2 and 3) as judged by MS and SDS-PAGE (Figure 3c,d). MS/MS analysis after 8 h of labeling confirmed modification of the target lysine within the LACE tag (Figure S5a). Besides the target lysine, minor modification was also observed at K7, which is a surface-exposed residue of AaLS-13 (Figure S5b) located in an 'inverted SUMOylation motif' that is known to be recognized by Ubc9.[22] Overall, control experiments with AaLS-13 cages that lack the external LACE tag showed no modification by intact MS, proving that modification at K7 was only present in trace amounts and the labeling site-specific (Figure S4c). Integrity of the biotinylated AaLS-13-LACE was verified by TEM

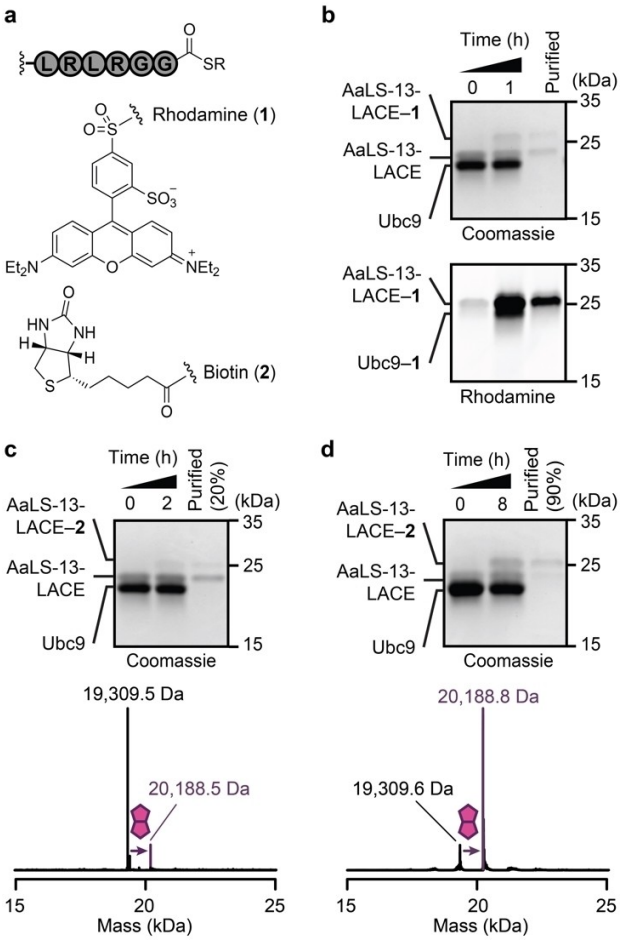

Figure 3. AaLS-13 cage modification with small molecule probes. (a) Structure of thioesters **1** and **2**. R = Ac-Cys-NHMe. (b) Coomassie-stained SDS-PAGE (top) and in-gel fluorescence (bottom) of AaLS-13-LACE labeling reactions with rhodamine thioester **1**. Coomassie-stained SDS-PAGE (top) and MS analyses of purified cages labeled with thioester **2** (bottom) after (c) 2 h and (d) 8 h. Mass differences and signals corresponding to biotinylated products are highlighted in purple.

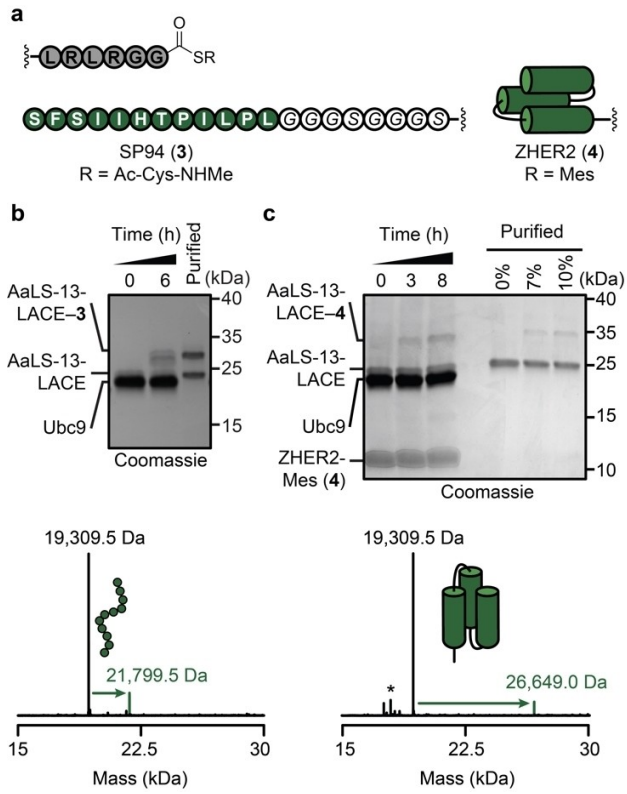

Figure 4. AaLS-13 cage modification with peptide and protein ligands. (a) Structure of thioesters **3** and **4**. Mes = 2-mercaptoethanesulfonate. SDS-PAGE (top) and MS analyses of purified cages (bottom) labeled with (b) thioester **3** and (c) thioester **4**. Mass differences and signals corresponding to labeled products are highlighted in green. The signal marked with an asterisk (*) corresponds to a minor degradation by-product resulting from cleavage of the LACE tag (PRAVIK*QESAE).

(Figure S4d). The ability of biotinylated AaLS-13-LACE cages to bind streptavidin on their exterior surface was assessed by native agarose gel electrophoresis (Figure S4e).

We next explored the potential of LACE to conjugate peptides to the surface of preassembled AaLS-13-LACE cages. As a model, we chose SP94, a 12-residue peptide that binds to the human GRP78 receptor.[23] SP94 has proven useful for targeting ferritin cages to hepatocellular carcinoma cells.[24] Thioester **3** was prepared by SPPS, and consists of the peptide SP94 equipped with a C-terminal extension (LRLRGG) separated by a flexible linker (Figure 4a, Table S1). Labeling under standard conditions (Table S2, Entry 4) resulted in approximately 45% conversion after 6 h as judged by SDS-PAGE analysis (Figure 4b). MS confirmed formation of the desired conjugate (Figure 4b), and SEC substantiated the integrity of the modified cages (Figure S6a). These results show that LACE can be readily used with synthetically accessible peptide thioesters for peptide display on AaLS-13.

As a test case for conjugation of a folded protein domain to pre-assembled cages, we chose the antibody mimic ZHER2, a HER2-targeting affibody.[25] The corresponding affibody thioester, with a C-terminal extension (LRLRGG), was prepared recombinantly from an intein fusion[26] by thiolysis with 2-mercaptoethanesulfonate (Mes) to afford ZHER2-Mes (**4**) (Figure 4a, Table S1). Reactions with ZHER2-Mes with AaLS-13-LACE under standard conditions proceeded to approximately 10% labeling after 8 h as judged by SDS-PAGE and MS (Figure 4c, Table S2, Entries 5,6). SEC and TEM analysis of the purified cages showed that labeling with the affibody domain did not disrupt the assembly (Figure S6b,c).

Having established modification of AaLS-13-LACE cages, we tested the generality of the approach by modifying the structurally distinct cage OP-LACE (Figures 5 and S7). OP-LACE was reacted with biotin thioester (**2**) and MS characterization of the purified cages indicated a conversion of 60% after 6 h (Figure S7a, Table S2, Entry 7). MS/MS analysis confirmed that the labeling occurred exclusively on the LACE tag (Figure S8), and control experiments with the OP cage lacking the displayed LACE tag showed that the parent protein was not modified by Ubc9 (Figure S7a). Labeling of OP-LACE with SP94 thioester **3** resulted in approximately 45% conversion (Figure 5a, Table S2,

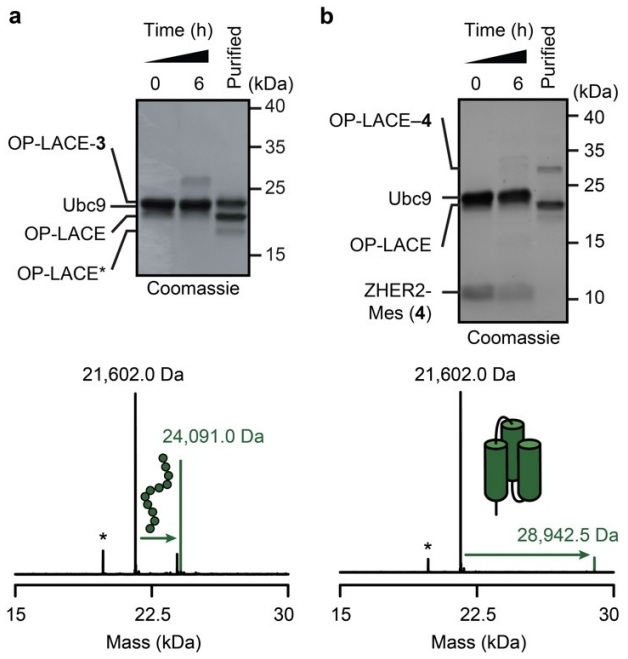

Figure 5. OP-LACE cage modification with peptide and protein ligands. SDS-PAGE (top) and MS analyses (bottom) of purified cages labeled with (a) thioester **3** and (b) thioester **4**. Mass differences and signals corresponding to labeled products are highlighted in green. The signal marked with an asterisk (*) corresponds to a minor degradation by-product resulting from cleavage of the LACE tag (PR*AVIKQESAE).

Entry 8), and reaction with ZHER2-Mes (**4**) proceeded to approximately 15% labeling after 6 h (Figure 5b, Table S2, Entry 9) as judged by SDS-PAGE and MS. SEC and TEM analyses confirmed that the OP-LACE cages remained intact following modification with SP94 and ZHER2 (Figure S7b–d). Despite the different size and symmetry of OP-LACE and AaLS-13-LACE, the achieved labeling efficiencies were comparable.

In conclusion, we have shown that LACE is a straightforward approach for modification of protein cages. Various thioesters, which are easily accessed synthetically or recombinantly, can be transferred irreversibly with a useful degree of monomer functionalization. This versatile conjugation technique could find application in designing new vaccine platforms, where modular methods for arraying antigens on multimerization scaffolds are highly sought after.[27] Additionally, LACE may enable engineering of delivery vehicles that target specific tissues and possess longer circulation half-lives.

Although the kinetics of Ubc9 are relatively slow, the extent of labeling could be conveniently controlled by adjusting the reaction time and the concentrations of substrate and enzyme. Because the reaction is irreversible, an excess of Ubc9 can be employed without causing hydrolysis of the conjugate. Ubc9 can be produced in *E. coli* in high yield and is easily removed from the cage products by SEC after labeling. Especially with ligands that are well tolerated by the cage structure, such as biotin, near quantitative modification can be achieved. In contrast, modification with the ligands SP94 and ZHER2 could not be driven to completion, which may be due to steric hindrance on the surface of the cages. However, we have previously demonstrated that AaLS-13 could be effectively targeted to specific cells when less than 3% of the subunits were functionalized with antibodies,[21] so substoichiometric labeling is unlikely to be problematic in most cases. Post-assembly modification of cages with LACE is suitable to achieve such defined degrees of labeling.

The LACE-equipped OP and AaLS-13 cages were equally well labeled, despite being very different in size and structure (Figures 2 and S1). In terms of reaction setup, the *in vitro* procedure allowed us to introduce a variety of synthetic and recombinant thioesters under controlled conditions. We have recently reported an engineered E1 enzyme that can activate and load ubiquitin onto Ubc9 in the cytoplasm of *E. coli*.[28] In the future, this E1 system could conceivably be used with an evolved Ubc9 variant to activate and conjugate desired peptide and protein moieties to cages *in cellulo*.

Given that LACE targets lysine side chains at internal sites, this method may be an attractive option for modification of protein cages that lack termini on their exterior surface.[29] Additionally, our approach leaves the N- and C- termini free for conjugation by other methods. For example, LACE is compatible with other protein labeling approaches, including sortase and SpyCatcher/SpyTag,[12] which may be exploited for one-pot dual modification of cages. Considering the broad cargo scope of AaLS-13 and OP, modification of these protein cages by LACE in a modular fashion and with control over the degree of labeling, each from a single recombinant precursor, has high potential for the development of molecular delivery and vaccine platforms.

Acknowledgements

We thank the Scientific Center for Optical and Electron Microscopy (ScopeM), ETH Zurich, for help with TEM experiments, and the Functional Genomics Center Zurich (FGCZ), ETH Zurich and University of Zurich, for mass spectrometry analysis. We are grateful to G. Akimoto for help with control experiments. D.H. was the recipient of an Innosuisse Innovation Grant (Project 42250.1). R.H. is grateful for a scholarship from the Stipendienfonds der Schweizerischen Chemischen Industrie (SSCI). Generous support from ETH Zurich and the Swiss National Science Foundation is also gratefully acknowledged. Open Access funding provided by Eidgenössische Technische Hochschule Zürich.

Conflict of Interest

The authors declare no conflict of interest.

Keywords: enzymes · LACE · lumazine synthases · protein cages · protein engineering

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Manuscript received: June 11, 2022
Revised manuscript received: August 9, 2022
Accepted manuscript online: August 11, 2022
Version of record online: September 15, 2022