Construction of a Self-Purification and Self-Assembly Coenzyme Regeneration System for the Synthesis of Chiral Drug Intermediates

Wei Jiang* and Wanru Zeng

ABSTRACT: As one of the important research contents of synthetic biology, the construction of a regulatory system exhibits great potential in the synthesis of high value-added chemicals such as drug intermediates. In this work, a self-assembly coenzyme regeneration system, leucine dehydrogenase (LeuDH)—formate dehydrogenase (FDH) protein co-assembly system, was created by using the polypeptide, SpyTag/SpyCatcher. Then, it was demonstrated that the nonchromatographic inverse transition cycling purification method could purify intracellular coupling proteins and extracellular coupling proteins well. The conversion rate of the pure LeuDH–FDH protein assembly (FR-LR) was shown to be 1.6-fold and 32.3-fold higher than that of the free LeuDH–FDH system (LeuDH + FDH) and free LeuDH, respectively. This work has paved a new way of constructing a protein self-assembly system and engineering self-purification coenzyme regeneration system for the synthesis of chiral amino acids or chiral α-hydroxy acids.

1. INTRODUCTION

Synthetic biology reveals its broad and promising application perspective in the industries of chemical synthesis, the production of energy, and medicine. Multi-enzymes to generate an artificial biocatalytic system have become a promising powerful tool to construct highly effective biocatalysts,4 but the constructions of multiple enzyme systems are rarely reported. Inspired by the natural multi-enzyme complexes that generate substrate channeling,2 constructing multi-enzyme complex systems is an ideal method to reinforce cofactor regeneration and chiral amino acids or chiral α-hydroxy acid production. Many molecular scaffolds, such as artificial scaffolds and protein scaffolds, have been constructed for the assembly of multi-enzyme complexes to enhance catalytic efficiency and signaling pathways.2,3 However, the process of scaffold construction and multi-enzyme coupling is complicated. Currently, Howarth et al. developed a genetically encodable and efficiently reactive SpyTag (peptide, 1.1 kDa)/SpyCatcher (protein, 12 kDa) pair by splitting the autocatalytic isopeptide bond generating the CnaB2 domain of Streptococcus pyogenes.4 After mixing, the SpyTag and SpyCatcher endure autocatalytic isopeptide bonding between Lys31 on SpyCatcher and Asp117 on SpyTag.5 The high specificity of this reaction and bio-compatibility of the peptide/protein conjugation make the SpyTag/SpyCatcher chemistry an ideal way for preparing self-assembly proteins with precise location. Elastin-like polypeptides (ELPs) are a synthetic genetically engineered protein polymer with elastic properties and high sensitivity to ambient temperature. Their sequence chosen for this work is based on the hydrophilic polypentapeptide (VPGXG)n, where X is a mixture of valine and glutamic acid.5 ELPs were used in protein purification and plasmid DNA separation as they have a reversible phase change process called the reverse transformation temperature (inverse temperature transition, ITT).5,6 Because the purification process is simple and fast, the ELP is used for purification of self-assembly proteins in this work.

In organic synthesis, chiral amino acids and chiral α-hydroxy acids are ideal catalysts, auxiliaries, and chiral starting materials.7 The unnatural amino acids, e.g., L-tert-leucine (l-Tle) as a precursor for pharmaceutically active compounds such as tumor-fighting agents or telaprevir (HCV) and drugs Reyataz (HIV) protease inhibitors,8 play a significant role in the food additive, agrochemical, cosmetic, and pharmaceutical industries as important and perspective building blocks.9 Since the biocatalytic methods with high enantioselectivity can be executed under mild conditions, it is promising to utilize biocatalyst to synthesize L-Tle. Meanwhile, chemical methods often require a rigorous process with a low production rate and poor chiral selectivity.10 The leucine dehydrogenase (LeuDH, EC 1.4.1.9) and formate dehydrogenase (FDH, EC 1.2.1.2) were selected for the synthesis of L-Tle by reductive amination of trimethylpyruvic acid (TMA) with the regeneration of expensive cofactor NADH.11 Cofactor regeneration is an ideal method for the coenzyme-dependent biosynthesis system.
as coenzymes are very expensive.\textsuperscript{11} Therefore, this system was selected as the research object for constructing a self-assembled cofactor regeneration system and producing chiral amino acids or chiral \( \alpha \)-hydroxy acids.

In this study, a self-assembled multi-enzyme cofactor regeneration system for \( l \)-Tle production was developed by using the SpyTag/SpyCatcher as irreversible spontaneous formation of isopeptide bonds of the SpyTag/SpyCatcher allows precise self-assembly of the LeuDH and FDH. In addition, ELPs were introduced in this system to obtain the self-purification coupling enzymes. The productions of \( l \)-Tle by this self-assembled bifunctional complex system and the free enzyme mixture were compared (Supporting Information Scheme 1).

2. RESULTS AND DISCUSSION

2.1. General Design. The ELP gene designed and used in this study is shown in the Supporting Information. Two types of functional fragment design method were examined: (1) heterotelechelic polymers (LeuDH−linker−SpyCatcher−ELPs, LR) that contains LeuDH, linker, SpyCatcher and ELPs (Figure 1) and (2) homopolymers (SpyTag−linker−FDH, FR) that contains SpyTag, linker, and FDH (Figure 1). The placement of the sequences encoding SpyCatcher within the protein-coding gene programs the post-translational modification of the target proteins in situ.\textsuperscript{4c} Inspired by this, SpyTag and SpyCatcher were placed at the N- and C-termini of the FDH and LeuDH, respectively, which led to proteins’ self-assembly, generating tadpole-like proteins (Supporting Information Scheme 1). Additionally, the ELPs were located with the LeuDH for purification of self-assembly proteins. Thus, each of these proteins could be expressed and purified separately. It was illustrated that the simplest examples could provide a proof of concept. These routes could be varied widely to give various H- or star-shaped proteins.\textsuperscript{4c} The full amino acid sequence for these proteins is listed in the Supporting Information.

2.2. Domain-Selective Self-Assembly to Tadpole Proteins in Extracellular and Living Cells. Accurate construction of macromolecules, such as synthetic polymers and multi-enzyme complexes, is challenging.\textsuperscript{4c} A polymer undergoes two types of reactions, cyclization and self-assembly, with SpyTag/SpyCatcher and ELP mutually reactive groups in polymer chemistry. Artificial scaffolds and protein scaffolds have been constructed for the assembly of multi-enzyme complexes to enhance the synthesis of polymer or tadpole-like proteins; however, the process is very complicated.\textsuperscript{2,3} It was demonstrated that SpyTag/SpyCatcher can be applied to construct unconventional protein topologies while these tadpole-, circular-, star-, H-shaped, and block proteins are inactive.\textsuperscript{4c} In living cells, an effective and simple route for synthesis of polymer or tadpole-like proteins can be obtained by using SpyTag/SpyCatcher with the adjustable assembly positions. The protein synthesis rate of a given expression host depends on the induction level (isopropyl-\( \beta \)-D-thiogalactoside concentration), culture medium, and temperature.\textsuperscript{13} It has been demonstrated that protein expression levels can be increased by increasing IPTG concentration and enhancing temperatures for \textit{Escherichia coli}.\textsuperscript{13a,13c} Therefore, plenty of heteroproteins or polymers would self-assemble and cyclize in situ. Additionally, this hypothesis was demonstrated by using SpyTag/SpyCatcher, generating tadpole-like FR, LR, and FR-LR in this work. These target proteins were assembled by using SpyTag/SpyCatcher, and the results were characterized by SDS-PAGE and are shown in Figure 2 (lane 5, Figure 2). In addition, FR and LR also could be connected in vitro, generating FR + LR with the same principle (lane 6, Figure 2).

2.3. Nonchromatographic Purification of a Domain-Selective Self-Assembly Enzyme Complex. The method described above provides an ideal and versatile route for the direct intracellular and extracellular biosynthesis of self-assembly proteins. It has advantages beyond the synthesis of a protein complex. Covalent closure of the amide backbone

![Figure 1](https://dx.doi.org/10.1021/acsomega.0c04668)  
Figure 1. Experimental design and process principles.

![Figure 2](https://dx.doi.org/10.1021/acsomega.0c04668)  
Figure 2. 10% SDS-PAGE analysis of recombinant \textit{E. coli} BL21 crude enzyme. M: protein maker; 1: LR; 2: FR; 3: FDH; 4: LeuDH; 5: FR-LR intracellular connection; 6: FR + LR extracellular connection.
enables the precise control over the self-assembly and formation of substrate channels to enhance the bioactivity and stability of circular peptides and proteins.\textsuperscript{4c} It is demonstrated that domain-selective cyclization gives many advantages in tailoring the protein’s properties.\textsuperscript{15} However, domain-selective self-assembly proteins have not yet been developed in protein engineering as far as we know. It was shown that the SpyTag/SpyCatcher was naturally suited for domain-selective cyclization to macromolecular proteins.\textsuperscript{4c} The domain flanked by SpyTag/SpyCatcher would be self-assembled when SpyTag was placed in the chain. The construction and purification of FR + LR and FR-LR were used to verify this idea. The target protein was purified by using ELPs, and the result was characterized by SDS-PAGE and is shown in Figure 3 (lane 7, Figure 3). In addition, FR and LR also could self-assemble in vitro, generating FR + LR with the same principle (lane 8, Figure 3). As can be seen from Figure 3, the purification effect of in situ self-assembly was better than that of expressed separately.

### 2.4. Kinetic Parameters.

Kinetic parameters of the pure FR + LR and FR-LR were calculated according to the Lineweaver–Burk double-reciprocal plot. Then, the result of kinetic parameters ($K_m$, $k_{cat}$, and $k_{cat}/K_m$) is summarized in Table 1. The $K_m$ value of the FR + LR for TMA was over 5 times than that of the FR-LR, and the $k_{cat}/K_m$ value of the FR-LR for TMA was 4.7 times higher than that of the FR + LR. The higher catalytic efficiency also improves the ability of its synthetic products as the conversion rate of the FR-LR was 1.6-fold and 32.3-fold than that of the LeuDH + FDH and LeuDH, respectively. These results indicated that the better substrate channels and closer electron transfer distances in synthetic cascades were critical to improve catalytic efficiency (Table 1) and enhance product yield (Figure 4).\textsuperscript{16} Other methods to synthesize l-Tle using LeuDH and FDH, such as individual expression, other ways of linking, and the double plasmids expression, were also constructed.\textsuperscript{12} A regular separate purification of the LeuDH and FDH followed by just mixing the two enzymes was executed, but it shows its flaws of laborious isolation, the two proteins were expressed once each, enzyme purification, and the enzymes with weak stability under these processes. The double plasmid expression in a whole cell takes twice as many antibiotics for enzyme expression, while only one plasmid was used to co-express the genes (FR-LR) for the l-Tle production in this work. Additionally, the bifunctional enzyme complex system can be purified by using ELPs with simple and quick steps. Therefore, the design idea of self-purification and self-assembly coenzyme regeneration system to form substrate channels and close electron transfer proximity could be potentially used as an ideal strategy to synthesize other crucial chiral drug intermediates and α-hydroxy acids.\textsuperscript{17}

### 3. CONCLUSIONS

In summary, it was demonstrated that the genetically encoded SpyTag/SpyCatcher chemistry could be applied to prepare a self-assembly coenzyme regeneration system. Additionally, the bifunctional enzyme complex system could be purified by using ELPs with simple and quick steps. The pure FR-LR had the efficiencies of l-Tle by LeuDH, LeuDH + FDH, FR + LR, and FR-LR were determined, and the results are shown in Figure 4. The e.e. value was considered more than 99% as D-Tle was not measured by HPLC analysis. The pure FR-LR exhibited the highest efficiency of the transformation of TMA into l-Tle, and the conversion rate of the FR-LR was 1.6-fold and 32.3-fold than that of the LeuDH + FDH and LeuDH, respectively. These results indicate that the better substrate channels and closer electron transfer distances in synthetic cascades were critical to improve catalytic efficiency (Table 1) and enhance product yield (Figure 4).\textsuperscript{12} Other methods to synthesize l-Tle using LeuDH and FDH, such as individual expression, other ways of linking, and the double plasmids expression, were also constructed.\textsuperscript{12} A regular separate purification of the LeuDH and FDH followed by just mixing the two enzymes was executed, but it shows its flaws of laborious isolation, the two proteins were expressed once each, enzyme purification, and the enzymes with weak stability under these processes. The double plasmid expression in a whole cell takes twice as many antibiotics for enzyme expression, while only one plasmid was used to co-express the genes (FR-LR) for the l-Tle production in this work. Additionally, the bifunctional enzyme complex system can be purified by using ELPs with simple and quick steps. Therefore, the design idea of self-purification and self-assembly coenzyme regeneration system to form substrate channels and close electron transfer proximity could be potentially used as an ideal strategy to synthesize other crucial chiral drug intermediates and α-hydroxy acids.\textsuperscript{17}

### Table 1. Apparent Kinetic Parameters

| enzyme       | substrate | $K_m$ (mM)       | $V_{max}$ (U⁻¹) | $K_{cat}$ (S⁻¹) | $K_{cat}/K_m$ (mM⁻¹ S⁻¹) |
|--------------|-----------|------------------|-----------------|-----------------|--------------------------|
| FR-LR (FR)   | COONH₄     | 22.50 ± 0.15     | 3.20 ± 0.12     | 377.62 ± 10.98  | 16.78 ± 0.72             |
| FR + LR (FR) | COONH₄     | 20.18 ± 0.98     | 1.67 ± 0.42     | 197.44 ± 0.47   | 9.78 ± 0.48              |
| FR-LR (LR)   | TMA        | 2.13 ± 0.33      | 13.97 ± 0.13    | 1497.95 ± 14.06 | 702.05 ± 4.26            |
| FR + LR (LR) | TMA        | 11.11 ± 0.41     | 16.35 ± 0.38    | 1676.23 ± 39.65 | 150.86 ± 9.61            |
highest conversion rate, displaying 1.6-fold and 32.3-fold than that of the free LeuDH + FDH and the LeuDH, respectively. These methods for engineering a self-purification coenzyme regeneration system and constructing protein self-assembly system provide an ideal platform for the exploitation of novel biomaterials for the synthesis of high-value-added chemicals.

4. EXPERIMENTAL SECTION

4.1. Strains, Chemicals, and Enzymes. The bacterial strains Escherichia coli DH5α (E. coli) was used as a host for DNA manipulation. E. coli BL21 (DE3) was used as the expression host. The recombinant bacteria were cultured in a Luria–Bertani medium (LB) with 50 μg/mL kanamycin at 37 °C. The competent cell preparation kit and all restriction enzymes used in the study were purchased from Takara Co., Ltd. (Dalian, China). The plasmid miniprep kit and gel DNA mini purification kit were provided by Omega China. In addition, all chemicals with analytical reagent or chromatographically pure were obtained from Shanghai Sangon Biotech Co., Ltd. (Shanghai, China). The functional fragments were synthesized by Shanghai Sangon Biotech Co., Ltd. (Shanghai, China).

4.2. DNA Construction. The ELPl gene was ordered from Shanghai Sangon Biotech Co., Ltd. (Shanghai, China). The SpyTag and SpyCatcher coding information were obtained from NCBI. The functional fragments, LeuDH–linker–SpyCatcher–ELPs (LR) and SpyTag–linker–FDH (FR), were designed in this work. All amino acid sequence information of the fragments are listed in the Supporting Information. The LR and FR were synthesized by Shanghai Sangon Biotech Co., Ltd. (Shanghai, China). The pET-28a-LR and pET-28a-FR were digested by double enzyme with BamHI and XhoI. After that, FR was linked with pET-28a-LR. After transformation, the positive plasmid was confirmed by sequencing to generate PET-28a-LR-FR. Then, the pET-28a-LR-FR was translated in DE3 pLysS cells to express the recombinant protein.

4.3. Protein Expression and Purification. The cells, which contain LeuDH, FDH, LR, FR, and LR-FR, were cultivated at 37 °C in 10 mL of LB (containing kanamycin) for overnight culture. Then, the cultured mixture was transferred to a 100 mL LB medium (containing 1:100 kanamycin) and cultured for 2–3 h at 37 °C until the optical density (OD600) reached 0.6–0.8. Then, the enzymes were induced with isopropyl thiogalactoside (IPTG, 0.1 mM) at 25 °C for overnight culture. After that, cells were harvested by high-speed centrifugation (15,000 rpm, 10 min). The cells were suspended with PBS buffer, and they were broken by ultrasonic cell disruption; the cell free extract was obtained by centrifugation (10,000 × g, 15 min). The enzyme was purified by a His-Trap HP affinity column according to prior protocols.18 The purities of obtained enzymes were characterized by SDS-PAGE, and the enzyme assays were conducted to measure the enzyme activities.

4.4. Enzyme and Protein Assays. Enzyme activity was determined by spectrophotometrically monitoring the changes in the NADH concentration during the reactions, which resulted in the changes in the absorbance at 340 nm (ε = 6220 M⁻¹ cm⁻¹) according to previous reports.38a One unit (U) of the enzyme activity was defined as the number of micromole enzyme catalyzing degradation or formation of 1 μmol NADH per min in the reductive amination or in the oxidative deamination with standard conditions.

4.5. Protein Coupling and ITC Purification. Proteins were dissolved in 50 mM PBS at pH = 7.0. Enzyme coupling was executed by mixing SpyCatcher and SpyTag solutions in appropriate stoichiometric ratios.3 The method of purifying proteins by using the inverse transition cycling (ITC) of ELPs can draw lessons from a previous report.13 Then, 0.1461 g/mL NaCl was included in the crude enzyme, and the mixture was transferred to a water bath for an incubation of 10 min at 37 °C. Then, the deposits were collected by centrifugation (13,000 rpm, 15 min). A moderate amount of pre-cold PBS buffer was added to dissolve the precipitation at an ice bath condition for 60 min. After that, the mixture was centrifuged (13,000 rpm, 15 min) to collect the supernatant. The above steps were repeated twice.

4.6. Production of l-Tle by Using a Self-Assembled Bifunctional Enzymes Complex. The cells of FDH, FR, LR, and FR-LR with LeuDH, LeuDH+FDH, and FR + LR as a control, which were employed to synthesize l-Tle from TMA, were inoculated, induced, cultivated, collected, centrifuged (10,000 × g, 10 min), washed with PBS buffer (0.2 M, pH 7.0), and collected. After that, the cells were re-suspended with NH₄H₂O–NH₄Cl buffer (1 M, pH 7.0), introduced into 6 mL reaction systems, and broken by ultrasonic cell disruption. The reaction conditions are as follows (6 mL): TMA (600 μL), ammonium formate (600 μL), NADH (300 μL), CaCl₂ (30 μL), and moderate cell-free extracts of E. coli cells in NH₄H₂O–NH₄Cl buffer (1 M, pH 8.0), 25 °C, 200 rpm. The samples (500 μL) were collected at 0, 0.5, 1, 2, 4, 6, 12, and 24 h then centrifuged (12,000 rpm, 6 min). After the termination of the reaction in the boiling bath, the mixture was centrifuged (12,000 rpm, 5 min) to collect the supernatant. The product was filtered via ion-exchange resin (Amberlite IR-120H) and determined by high-performance liquid chromatography (HPLC). The detected conditions are as follows: column, Chirex3126, Phenomenex; eluent, 2% CuSO₄ aqueous solution/isopropanol = 95/5; flow rate, 1.0 mL/min.15

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.0c04668.

(Scheme 1) Schematic illustration of construct encoding FR-LR, (Figure S1) double bottom figure of apparent kinetic parameters, and all amino acid sequences information of the fragments (PDF)

AUTHOR INFORMATION

Corresponding Author

Wei Jiang — College of Chemical Engineering, Huaqiao University, Xiamen, Fujian 361021, China; orcid.org/0000-0002-8194-6003; Phone: +86-05926162305; Email: wjiang@hqu.edu.cn, tianya416@126.com; Fax: +86-05926162305

Author

Wanru Zeng — College of Chemical Engineering, Huaqiao University, Xiamen, Fujian 361021, China

Complete contact information is available at: https://pubs.acs.org/10.1021/acsomega.0c04668
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