Chemical Protein Synthesis

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Ligation of Soluble but Unreactive Peptide Segments in the Chemical Synthesis of *Haemophilus Influenzae* DNA Ligase

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Abstract: *During the total chemical synthesis of the water-soluble globular Haemophilus Influenzae DNA ligase (Hin-Lig), we observed the surprising phenomenon of a soluble peptide segment that failed to undergo native chemical ligation. Based on dynamic light scattering and transmission electron microscopy experiments, we determined that the peptide formed soluble colloidal particles in a homogeneous solution containing 6 M guanidine hydrochloride. Conventional peptide performance-improving strategies, such as installation of a terminal/side-chain Arg tag or O-acyl isopeptide, failed to enable the reaction, presumably because of their inability to disrupt the formation of soluble colloidal particles. However, a removable backbone modification strategy recently developed for the synthesis of membrane proteins did disrupt the formation of the colloids, and the desired ligation of this soluble but unreactive system was eventually accomplished. This work demonstrates that an appropriate solution dispersion state, in addition to good peptide solubility, is a prerequisite for successful peptide ligation.*

Introduction

Chemical protein synthesis, wherein peptide segments prepared by solid-phase peptide synthesis (SPPS) are covalently linked by chemoselective ligations (in particular, native chemical ligation (NCL)), enables the preparation of proteins that are difficult to obtain by recombinant expression such as post-translationally modified proteins and mirror-image proteins.[1,2] Early studies in this field were hampered by the difficulties associated with accessing the requisite ligation segments (for example, peptide thioesters),[3] but the intensive study of methods such as *N*-acyl-benzimidazolinone or hydrazide-based NCLs, microwave or flow-based SPPS, and many one-pot or sequential multisegment ligation strategies,[4,5] has enabled chemical syntheses of relatively large proteins such as a 312-residue GroEL/ES dependent protein,[6] the 352-residue *Sulfolobus solfataricus* P2 DNA polymerase IV (Dpo4)[7] and a hexa-ubiquitin containing 456 amino acids.[8] These accomplishments did not reveal limitations in NCL and instead suggested that any small-to-medium-sized protein should be readily amenable to chemical synthesis, assuming the availability and good solubility of its constituent peptide segments. Herein, we address this confidence with the disclosure of a highly soluble and readily accessible peptide segment that did not undergo ligation using standard NCL conditions.

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Results and Discussion

Convergent synthesis of DNA ligase Hin-Lig

Our discovery was made during the study of biochemical systems comprising homochiral D-amino acids and L-nucleic acids, the construction of which may enable the production of enantiomeric biomacromolecules for drug discovery[9] and yield clues as to the origin of life.[10] Chemical protein synthesis is well-suited to this task; for example, Kent et al. synthesized a D-form HIV-1 protease (99 aa) with enzymatic specificity toward the D-peptides,[11] and we synthesized the D-form of African swine fever virus polymerase (174 aa), which we used to achieve mirror-image DNA replication and RNA transcription.[10] To amplify L-DNA by polymerase chain reactions and to achieve reverse transcription, we also synthesized enantiomeric Dpo4 (352 aa).[7c] The next step in these studies was the construction of the requisite mirror-image DNA ligase needed for the preparation of larger L-DNA fragments. To this end, we designed a convergent synthetic strategy using hydrazide-based NCL for the synthesis of the 268-residue *Haemophilus Influenzae* DNA ligase (Hin-Lig).

The target protein was divided into six peptide segments (**1**–**6**) that contained 23, 58, 49, 33, 56, and 55 residues, respectively (Figure 1 A). For the purposes of NCL, five Cys residues were temporarily installed at positions 18, 76, 125, 158, and 214; it was planned to convert these back to Ala after the ligations had been completed.[12] A His_6-tag was added to

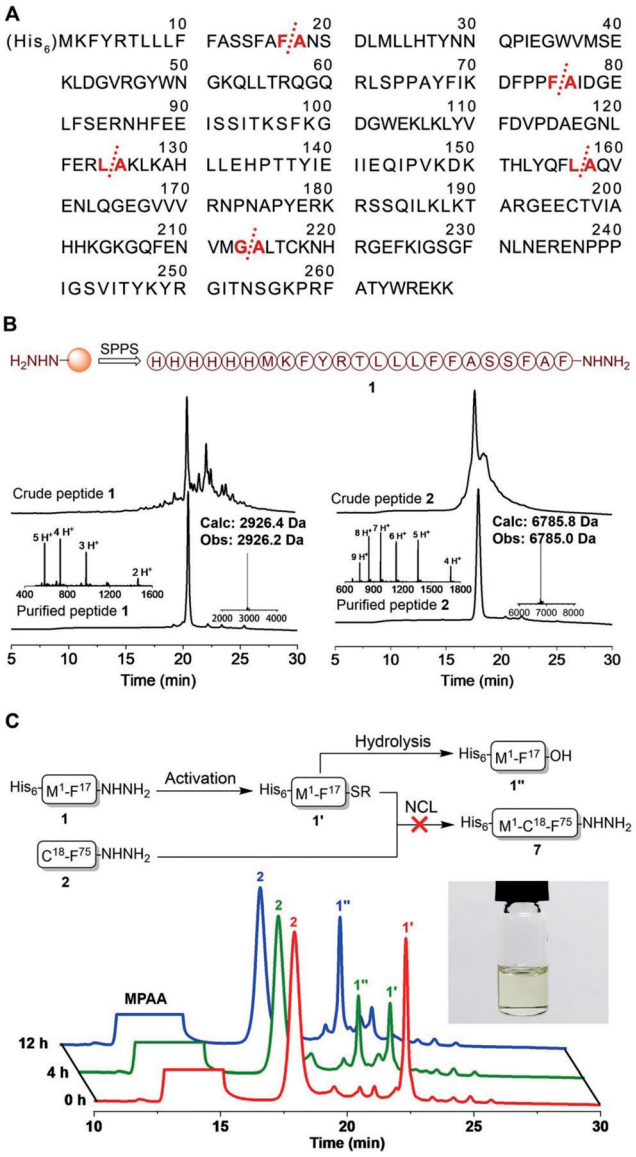

Figure 1. A) The amino acid sequence of His_6-Hin-Lig. The full-length protein is divided into six segments (shown by dashed lines) and the ligation sites are highlighted in bold. B) Synthesis of peptide **1** by SPPS; analytical HPLC chromatogram ($\lambda = 214$ nm) and ESI-MS spectra (including deconvoluted spectra) of the crude and purified peptides **1** (left) and **2** (right). C) Analytical HPLC traces ($\lambda = 214$ nm) for the ligation of **1** with **2** for 0, 4, and 12 h. Key: peptide thioester of **1** (**1′**), hydrolyzed product of **1′** (**1″**).

the N-terminal of Hin-Lig to facilitate the purification of the final protein product.

Peptide segments **1–6** were readily prepared by SPPS based on the 9-fluorenylmethyloxycarbonyl (Fmoc) strategy, identified by electrospray ionization mass spectrometry (ESI-MS), and purified by reversed-phase HPLC with good isolated yields (10.4%, 8.4%, 7.0%, 22%, 8.5%, 8.6%, respectively). To facilitate the ligation, the N-terminal Cys of peptides **4** and **5** were protected as 3-(trifluoroacetyl)-1,3-thiazolidine-4-carboxylic acid (Tfa-Thz).[13] Furthermore, Cys^{196} and Cys^{217} were protected with acetamidomethyl (Acm) to enable the ligation-desulfurization strategy.

With peptides **1–6** in hand, we carried out the condensation reactions using standard hydrazide-based NCL.[14] The first ligation was conducted between **1** (1.0 equiv, 0.6 mM) and **2** (1.5 equiv, 0.9 mM) and was expected to be straightforward because **1** and **2** were both water-soluble peptides that were easily made and characterized (Figure 1B). After $NaNO_2$ activation at pH 3, we detected clean conversion of the hydrazide **1** to the 4-mercaptophenylacetic acid (MPAA) thioester **1′**. The subsequent ligation was attempted at pH 6.5, and at first appeared to resemble a well-behaved NCL system without any turbidity (Figure 1C). Surprisingly, no desired ligation product **7** could be detected from the reaction even after several repeats; complete hydrolysis of **1′** to **1″** took place instead. This behavior was very puzzling, since it has been repeatedly established that NCL is faster than hydrolysis, provided the solubility of the NCL partners is good.[1,4] More detailed analysis showed that about half of the peptide thioester **1′** remained intact after the ligation was conducted for 4 hours (Figure 1C), whereas the Phe-Cys ligation could reach 90% conversion within 4 hours.[1b] Therefore, the reason for the failure of NCL of **1′** is that the ligation was slowed down.

Our initial response to this problem was to attempt the ligation at even higher concentrations to speed up NCL. To increase the solubility of peptide **1**, we turned to the polyarginine tag method specifically invented to increase the solubility of peptides.[15] Peptide **1a** was synthesized by appending the Arg_4 tag to the N terminus of **1** (Figure 2A). Using nanodrop spectrophotometry at 280 nm, the solubility of **1a** was determined to be as high as 8 mM in a membrane-filtered aqueous solution containing 6 M guanidine hydrochloride (Gn·HCl). Subsequently, the hydrazide-based ligation of **1a** (2.5 mM) and **2** (2.5 mM) was attempted. Surprisingly, very little target product **7a** was observed; the dominating reaction was once again hydrolysis of **1a** to **1a″**. To speed up ligation further, we tried addition of organic co-solvents (that is, 30% acetonitrile or 20% 2,2,2-trifluoroethanol) to the ligation system, but failed to see any improvement (Supporting Information, Figure S1). At this point, we concluded that the desired NCL was hindered by poorly recognized factors and further investigation would be needed.

Formation of soluble colloidal particles

To pinpoint which of the two peptides (**1a** and **2**) was the source of the problem, each was separately ligated with two other peptides (hydrazide **M1** and N-Cys peptide **M2**) previously shown to have good ligation activity[7a] (Supporting Information, Figure S2). It was found that the ligation between **M1** and **2** proceeded smoothly, but the ligation between **1a** and **M2** failed and only the hydrolysis byproduct **1a″** was obtained. Accordingly, we reach the perplexing conclusion that the problem stemmed from **1a**—even though we had previously found this peptide to be extremely soluble (8 mM). After contemplating this problem for a long time, we made the serendipitous observation that a laser pointer

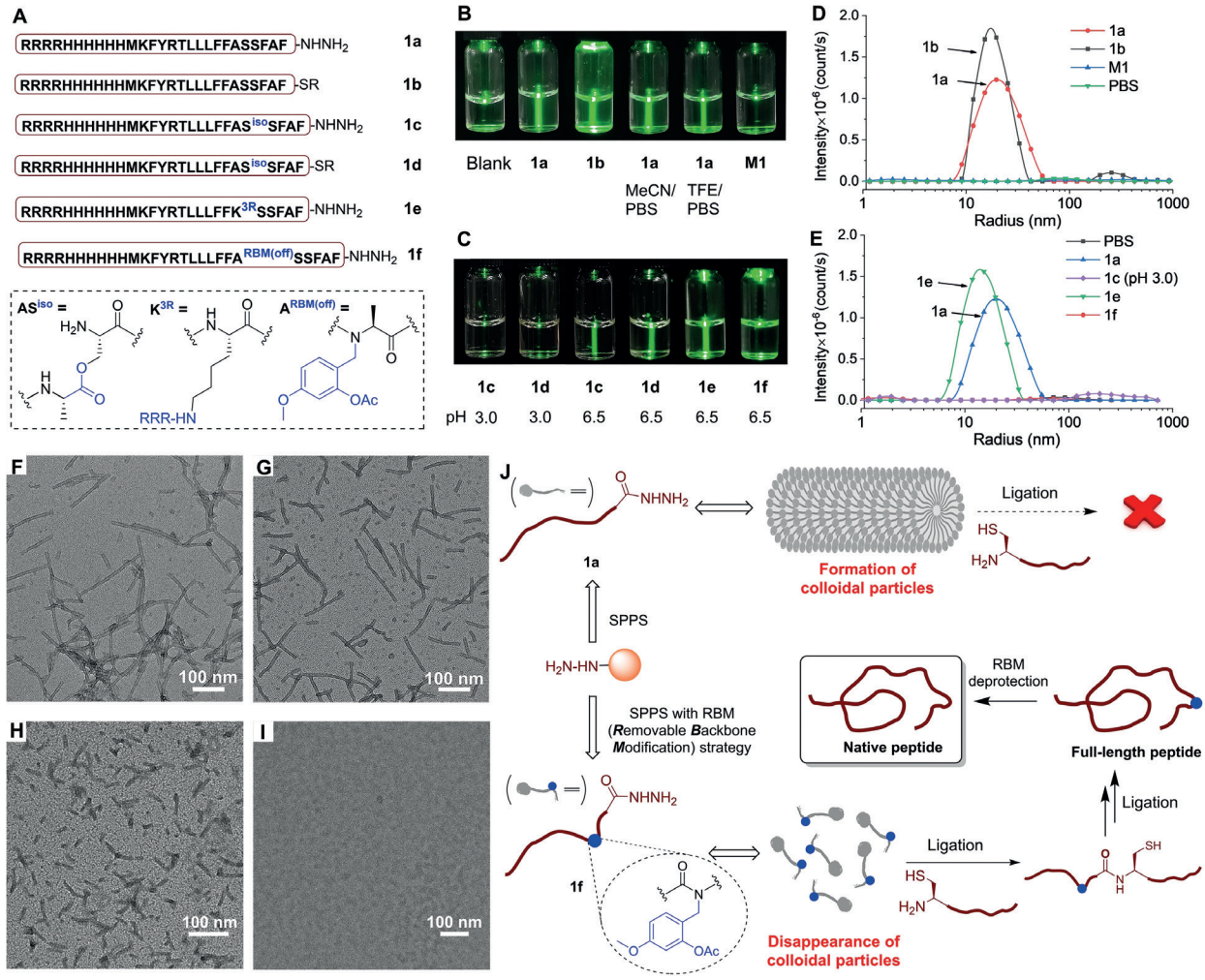

Figure 2. A) Amino acid sequences of peptide **1** and its modified analogues. B,C) The Tyndall effect of peptide **1** and its modified analogues. The tests were performed in the ligation buffer (0.1 M phosphate, 6 M Gn·HCl, pH 4.5) if not specially described; phosphate-buffered saline (PBS), acetonitrile (MeCN), 2,2,2-trifluoroethanol (TFE). D,E) Hydrodynamic radius distributions of blank, peptides **1a**–**1f**, and **M1** in the ligation buffer. F–I) TEM images of **1a** (F), **1b** (G), Arg_3-tagged peptide **1e** (H), and peptide **1f** prepared by the RBM strategy (I). J) Principle of soluble but unreactive peptide and peptide ligation by the RBM strategy.

(ca. 530 nm) could illuminate an aqueous Gn·HCl (6 M) solution of **1a** (an observation subsequently attributed to the Tyndall effect), but not an aqueous solution of Gn·HCl on its own (Figure 2B). A similar Tyndall effect was also observed with a solution of 2-mercaptoethanesulfonate sodium (MES-Na) thioester **1b** (obtained from **1a**), and/or when organic co-solvents (that is, 30% acetonitrile or 20% 2,2,2-trifluoroethanol) were added. Finally, no Tyndall effect was noted for the solution of peptide **M1**. As the Tyndall effect is predicated on the existence of colloidal particles, this led to the conclusion that peptide **1a** had indeed formed colloidal particles, which was highly unexpected for two reasons: 1) peptide aggregation usually leads to poor solubility, yet peptide **1a** had been previously established to be highly soluble, and 2) peptides dissolved in 6 M Gn·HCl are not anticipated to retain their secondary structures.

To confirm the formation of the colloidal particles, and also to analyze their size and morphology, solutions of peptides **1a** and **1b** in ligation buffer were examined by dynamic light scattering (DLS) and transmission electron microscopy (TEM). DLS spectra of solutions of **1a** (1 mM) and **1b** (1 mM) exhibited large peaks at about 20 nm. In comparison, the signal intensity of a solution of the peptide **M1** was very low and almost identical to that of the blank aqueous Gn·HCl (6 M) solution (Figure 2D). Meanwhile, the TEM imaging of **1a** and **1b** showed that both peptides had self-assembled to form rod-like, non-hollow nanostructures with diameters of about 10 nm and lengths of 100–200 nm (Figures 2F,G). Computational modeling of **1a** and **1b** indicated their stretch lengths were about 5 nm. Therefore, the structures observed by TEM were interpreted to be cylindrical micelles. The hydrodynamic radii of these cylindrical micelles were about 20 nm, according to the DLS measurement. Although similar cylindrical micelles have been previously described for some peptides that self-assembled under physiological conditions,[16] our present results demonstrate that such structures can be stable in a homogeneous solution containing 6 M Gn·HCl. The present

finding is therefore highly significant as it highlights that, in addition to peptide solubility, the solution dispersion state is also necessary for successful peptide ligation.

To examine the effect of concentration on the formation of the colloidal particles, we measured the DLS signals of solutions of **1a** in aqueous Gn·HCl (6 M) of different concentrations. The lowest concentration at which we could still observe the DLS signals was determined to be 2.3 μM, which was presumably the critical micelle concentration of **1a** in the ligation buffer (Supporting Information, Figure S3). This concentration was three orders of magnitude lower than the concentration of **1a** at which NCL was first attempted (ca. 1 mM), and therefore explained why the ligation of **1a** failed to take place.

Methods to overcome the problem

Having identified the source of the problem, a solution to it was sought, and different strategies previously developed to improve the performance of peptide ligation were considered. The attachment of an N-terminal Arg tag to **1a** and addition of organic solvents to its solutions do not impede the formation of soluble colloidal particles and/or effect the ligation. Subsequently, we tested the *O*-acyl isopeptide strategy that was developed to improve peptide handling properties.[17] Thus, peptide **1c** bearing an isopeptide unit at Ala^{12}-Ser^{13} was prepared (Figure 2A) and dissolved in the buffer (6 M Gn·HCl, pH 3.0). It was found that **1c** did not exhibit the Tyndall effect (Figure 2C) or give rise to any absorption in DLS (Figure 2E). TEM measurements revealed that solutions of **1c** were devoid of colloidal particles (Supporting Information, Figure S4). Accordingly, we attempted the ligation by first activating **1c** with $NaNO_2$ at pH 3.0, followed by ligation with **2** at 6.5. The desired product **7a** was observed by analytical HPLC but in very low yield (19%; Supporting Information, Figure S5A). To investigate why the ligation was still poor, we examined the solution properties of **1c** and its corresponding MESNa thioester **1d**. At pH 3.0, the two solutions exhibited no Tyndall effect. However, after changing the pH of the solutions to 6.5 and allowing them to stand for 2 hours, both **1c** and **1d** showed a strong Tyndall effect, presumably because of their conversion from the isopeptide form to the native peptide form. Accordingly, further study of the *O*-acyl isopeptide strategy was abandoned.

Another strategy to assist peptide ligation was to attach a temporary solubilizing tag into amino acid side chains such as Glu, Lys, Thr, or Cys.[18] To test this strategy for the soluble but unreactive systems, we mutated Ala^{12} in **1a** to Lys^{12} and attached it to an Arg_3 tag to give peptide **1e** (Figure 2A). Peptide **1e** was found to be highly soluble in ligation buffer at both pH 3.0 and 6.5, but also exhibited a significant Tyndall effect (Figure 2C) and a large absorption peak indicative of colloidal particles of a radius of about 14 nm in the DLS spectrum (Figure 2E). In the TEM imaging experiment, we also observed cylindrical nanostructures with a diameter of about 10 nm and a length of between 20 and 70 nm (Figure 2H). These results suggested that attachment of the solubilizing tag had failed to impede formation of soluble colloidal particles. Consistent with this conclusion was the very low yield of product **7b** (12% by HPLC) obtained upon attempting the ligation of **1e** (2.5 mM) and **2** (2.5 mM) (Supporting Information, Figures S5 and S6).

Finally, we tested the strategy of removable backbone modification (RBM) that was recently developed to assist the ligation in the synthesis of membrane proteins[19] (Figure 2J). In this strategy, a temporary backbone amide N-modification group is incorporated into the peptide prior to ligation, the function of which was previously thought only to increase peptide solubility. Herein, we incorporated a RBM^{off} group (2-acetoxy-4-methoxybenzyl)[20] between Phe^{11} and Ala^{12} of **1a** resulting in **1f**. This RBM^{off} group is known to be stable to trifluoroacetic acid (TFA), and therefore, we could readily obtain peptide **1f** (11% isolated yield) from Fmoc SPPS. Gratifyingly, a solution of **1f** in ligation buffer at pH 6.5 did not exhibit a Tyndall effect (Figure 2C) and its DLS spectrum was identical to that of a blank buffer (Figure 2E). Moreover, no colloidal particles were observed in TEM images of **1f** (Figure 2I). Encouraged by these observations, we attempted hydrazide-based NCL of **1f** (2.5 mM) and **2** (2.5 mM) to give the desired product **7c** (Figure 3A). The reaction was completed within 12 hours by analytical HPLC monitoring to give the desired product as the only main peak (Figure 3B; 43% isolated yield) and without any noticeable hydrolysis. The HPLC trace obtained in this reaction and depicted in Figure 3B is in stark contrast to that obtained from the reaction depicted in Figure 1C (where the ligation yield was almost zero), emphasizing the efficacy of the RBM strategy. Notably, during the ligation the RBM^{off} group (2-acetoxy-4-methoxybenzyl) was hydrolyzed to RBM^{on} (2-hydroxy-4-methoxybenzyl), which was susceptible to removal by TFA and therefore could easily be removed in the final stage of synthesis, and after all the ligations. Collectively, these results establish that the RBM strategy could provide a useful solution to the difficulties associated with NCL in soluble but unreactive systems that are susceptible to colloid formation.

Successful synthesis of bioactive Hin-Lig

The aforementioned ligation between **1f** and **2** gave **7c**. Subsequent ligation of **7c** with segment **3** (1.2 equiv) was clean and produced the left half of the target protein (that is, **8a**) with an isolated yield of 44% (Figure 3B). Meanwhile, the right half of the target protein (that is, **10**) was prepared by the C-to-N sequential ligation.[21] The hydrazide-based NCL between **5** and **6** was performed at pH 6.5 for 17 hours and the pH value was then raised to 8.0 to remove the Tfa group. Subsequently, the reaction system was treated with $MeONH_2$ at pH 4 for 3 hours to produce **9** in 40% isolated yield.[22] In a similar fashion, the ligation of **9** and **4** (1.2 equiv) was conducted, which led to production of **10** in 56% isolated yield after Tfa (that is, 3-trifluoroacetyl) deprotection and Thz-to-Cys conversion. Finally, the left half **8a** was converted to a peptide MESNa thioester **8b** (59% isolated yield) through activation and thiolysis, which was reacted with the right half **10** for 18 hours to form a full-length peptide **11a**

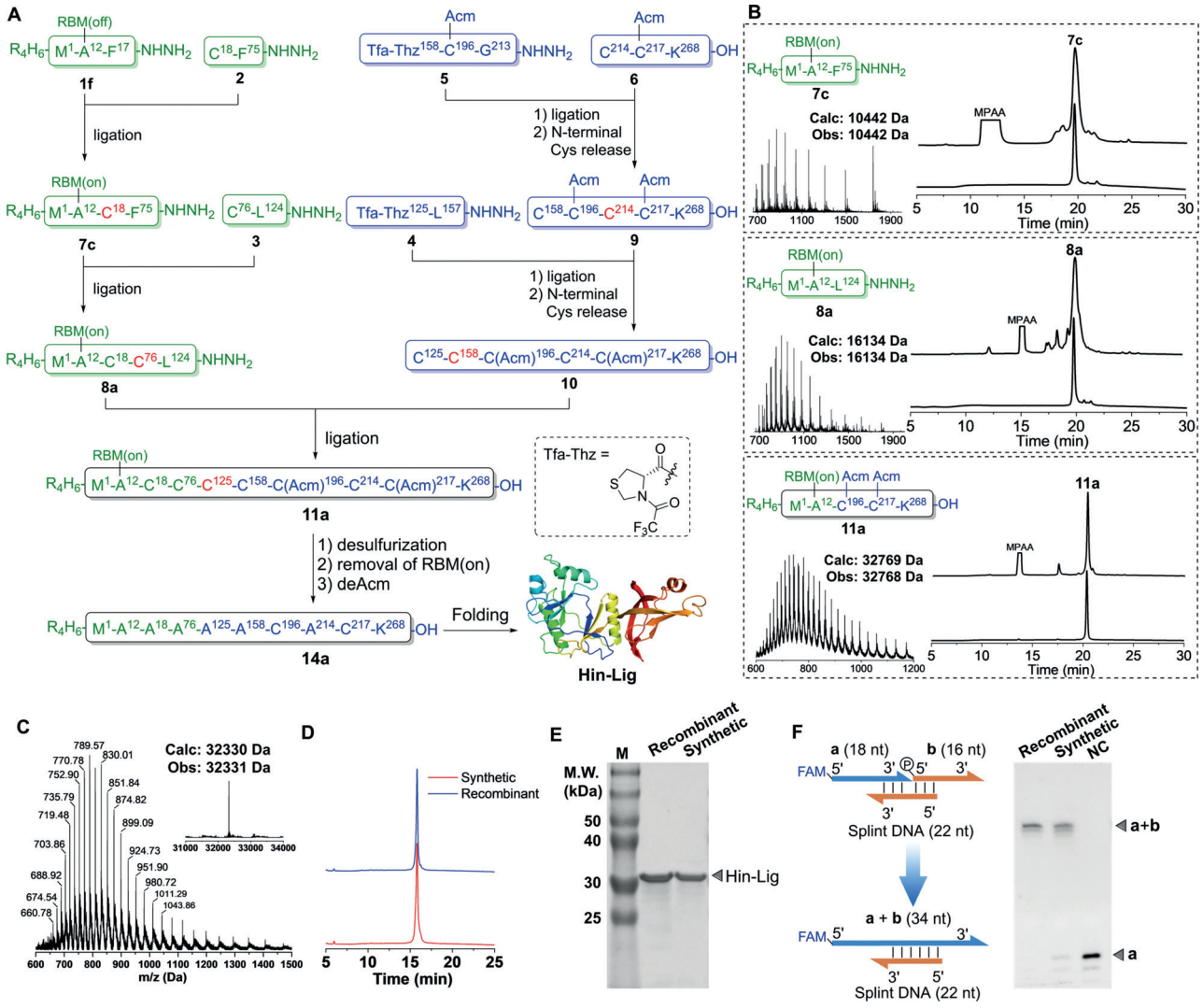

Figure 3. Chemical synthesis and characterizations of Hin-Lig. A) Synthetic route for Hin-Lig. B) Analytical HPLC traces of the ligations and the HPLC traces and ESI-MS spectra of the purified ligation products (**7c**, **8a**, and **11a**; Supporting Information, Figures S18, S19, and S23). C) ESI-MS and deconvoluted spectra of synthetic Hin-Lig **14a**. D) Analytical HPLC data of synthetic (red) and recombinant (blue) Hin-Lig. E) SDS-PAGE analysis of synthetic and recombinant Hin-Lig stained by Coomassie Brilliant Blue; protein marker (M). F) The DNA ligation assay of folded Hin-Lig and its analysis by PAGE in 8 M urea. The 5′-FAM-labeled DNA substrate **a** was ligated to the 5′-phosphate substrate **b** by recombinant and synthetic Hin-Lig; negative control with DNA substrates, ligase buffer, and refolding buffer, but without Hin-Lig (NC).

smoothly and cleanly in 68 % isolated yield (Figure 3 B). Free-radical-based desulfurization of **11a** was conducted using a soluble phosphine and radical initiator (VA-044/TCEP) for 12 hours, which converted all the five ligation-site Cys residues back into Ala in 57 % isolated yield.[12b] The product **12a** was dissolved in TFA to remove the RBM^{on} group within 3 hours to afford **13a**.[19a,20c] Finally, the S-Acm groups on Cys^{196} and Cys^{217} were removed by $PdCl_2$,[23] leading to the full-length peptide **14a** in 72 % isolated yield. The high-resolution ESI-MS and analytical reverse phase HPLC spectra of synthetic **14a** and recombinant Hin-Lig were identical (Figures 3 C,D). Moreover, synthetic **14a** was characterized by the SDS-PAGE electrophoresis method, which gave a single band at the expected molecular weight of approximately 32 kDa—the same as that of recombinant Hin-Lig (Figure 3 E). To further verify the amino acid sequence of **14a**, peptide mapping was performed by liquid chromatography tandem mass spectrometry (LC-MS/MS). The trypsin and pepsine digestions led to sequence coverages as high as 98.2 % (Supporting Information, Table S1). These sequence coverages were fully consistent with that of recombinant Hin-Lig.

The full-length peptide **14a** was folded by 100-fold dilution from aqueous Gn·HCl (6 M) solution into the renaturation buffer and its in vitro DNA ligation activity was evaluated. After the annealing of 5′-carboxyfluorescein (5′-FAM)-labeled acceptor oligonucleotides (18 mer, **a**), 5′-P donor oligonucleotides (16 mer, **b**) and complement DNA (22 mer), the DNA ligation reaction was initiated by the addition of T3 DNA ligase buffer and synthetic or recombinant Hin-Lig. The mixture was incubated for 1 hour at 22 °C. The ligation DNA products were analyzed by 20 % PAGE in 8 M

urea. The result showed that the synthetic and recombinant Hin-Lig gave the same linked product, indicating that synthetic ligase can efficiently ligate single-strand DNA (Figure 3F). Thus Hin-Lig prepared through total chemical synthesis exhibited good bioactivity and would be useful for the development of mirror-image biochemical systems. During the course of this study, Weidmann et al. reported the synthesis of a mirror-image, truncated version of Hin-Lig, lacking 16 amino acids at the N-terminus.[24] That the truncated version functioned as an effective ligase is interesting given the difficulties associated with the ligation of the N-terminal peptide segment.

Conclusion

In summary, we have established that: 1) good solution dispersion is an essential prerequisite for NCL, 2) consideration of solubility alone is inadequate, 3) the Tyndall effect exhibited by colloidal peptides is a good predictor of NCL failure, and 4) RBM of a peptide otherwise prone to aggregate in solution could provide an effective method of disrupting this aggregation. These discoveries were made in the course of our efforts to synthesize the Hin-Lig, which were ultimately successful.

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Conflict of interest

The authors declare no conflict of interest.

Stichwörter: DNA-Ligase · Native chemische Ligation · Proteine · Rückgratmodifikation · Spiegelbildliche Biologie

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