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Peptide Ligation

Selective Peptide Cysteine Manipulation on Demand and Difficult Protein Chemical Synthesis Enabled by Controllable Acidolysis of *N,S*-Benzylidene Thioacetals

Hongxiang Wu[+], *Zhenquan Sun*[+], *and Xuechen Li**

Abstract: Although solid-phase peptide synthesis combining with chemical ligation provides a way to build up customized polypeptides in general, many targets are still presenting challenges for the conventional synthetic process, such as hydrophobic proteins. New methods and strategies are still required to overcome these obstacles. In this study, kinetic studies of Cys/Pen ligation and its acidolysis were performed, from which the fast acidolysis of substituted *N,S*-benzylidene thioacetals (NBTs) was discovered. The study demonstrates the potential of NBTs as a promising Cys switchable protection, facilitating the chemical synthesis of peptides and proteins by efficiently disrupting peptide aggregation. The compatibility of NBTs with other commonly adopted Cys protecting groups and their applications in sequential disulfide bond formation were also investigated. The first chemical synthesis of the native human programmed death ligand 1 immunoglobulin V-like (PD-L1 IgV) domain was achieved using the NBT strategy, showcasing its potential in difficult protein synthesis.

Introduction

Simple and effective synthetic methods such as click chemistry[1,2] dramatically minimize sophisticated operations and expand the diversity of the end-users, by lowering the requirement of know-how knowledge.[3–5] As exemplified by the advent of solid phase peptide synthesis (SPPS),[6–8] its development began making peptide molecules readily available to almost anyone from different fields. The development of chemoselective peptide ligation is another advancement which can chemoselectively join the unprotected peptide C-terminal surrogates with the N-terminal amino acid or its surrogate of another peptide together.[9–14] Combining them further simplifies the access to peptides and proteins with customized modifications in atomic level precision.[15–17] These chemically synthesized peptides/proteins in homogeneous forms provide unique opportunity to unveil the role of site-specific post-translational modifications (PTMs) in fundamental chemical biology and to develop the next-generation peptide/protein-based therapeutic modalities.[18,19] Albeit these developments, there are still many important peptides/proteins not directly achievable by simple SPPS and peptide ligation, requiring complicated assisting strategies and sometimes even compromising detour of residue mutation for improving their synthetic performance.[20,21] Establishing more simple and general strategy to tackle those challenges is still of continued interest.

Previously, our group developed the serine/threonine ligation (STL)[11,12] and cysteine/penicillamine ligation (CPL)[14] for effective synthesis of peptides and proteins. Briefly, an unprotected peptide fragment with C-terminal salicylaldehyde (SAL) esters undergoes chemoselective ligation with the N-terminal Ser/Thr/Cys/Pen of the other unprotected peptide to form the *N,O*-benzylidene acetal (NBA) for STL or *N,S*-benzylidene thioacetal (NBT) for CPL by imine capture, oxazolidine/thiazolidine formation and *O*/*S*-to-*N* acyl transfer.[19,22] After treating with trifluoroacetic acid (TFA) cocktail, both NBA and NBT can be acidolyzed to produce the native peptide bond at the ligation site, albeit for NBTs much harsher conditions and longer time are required.[14] Till now both STL and CPL have been successfully applied for the chemical syntheses of various valuable peptides/proteins including immune checkpoint proteins,[23] cytokines,[24,25] nucleosome-associated proteins with PTMs,[26–30] antimicrobial cyclic peptides,[31–34] etc. Importantly, the generated NBA/NBT intermediate during STL/CPL becomes advantageous in challenging protein synthesis since the highly twisted structure of NBA and NBT can serve as an aggregation disruptor, which allows us to complete the first chemical synthesis of the immune

[*] H. Wu,[+] Z. Sun,[+] X. Li
Department of Chemistry,
State Key Laboratory of Synthetic Chemistry,
The University of Hong Kong, Hong Kong, SAR, People's Republic of China
E-mail: xuechenl@hku.hk
X. Li
Laboratory for Marine Drugs and Bioproducts,
Qingdao National Laboratory for Marine Science and Technology,
Ocean University China
Qingdao 266237, People's Republic of China
[+] These authors contributed equally.

checkpoint protein programmed cell death protein 1 (PD-1) extracellular domain.[23] However, the native *N,S*-benzylidene thioacetal (H-NBT) is so stable that the deprotection of multiple H-NBTs at one time may take very long time and need sophisticated handling. In some ligation sites (e.g. Pro-Pen), TFA/EDT/TMSOTf conditions would be necessary for acidolysis of one NBT structure. This shortage limits the multiple incorporation of NBTs in one synthesis which would be potentially advantageous for challenging targets.

To address the above issues, we performed the kinetic studies of Cys/Pen ligation and its acidolysis at various ligation sites with different substituted salicylaldehydes. Unexpectedly, the rapid acidolysis of methoxy-substituted *N,S*-benzylidene thioacetals (MeO-NBTs) which finished in minutes was discovered. We further demonstrated that such advantageous feature could make substituted NBTs as a promising Cys switchable protection which could be introduced in early/late stage of synthesis on demand and easily removed with fast kinetics even in multiples (Figure 1). At the same time by blocking the adjacent amide proton and forming a twisted conformation, the NBT protection could efficiently disrupt peptide aggregation to facilitate the chemical syntheses of peptides and proteins. To this end, one-pot acidolysis of multiple MeO-NBTs in peptide and its tandem usage with superfast desulfurization have been examined. Moreover, the compatibility of NBTs with other commonly adopted Cys protecting groups was investigated. In addition, the NBT-involved sequential disulfide bond formation was also evaluated including the utilization of distinct kinetics between different substituted NBTs. Furthermore, the chemical synthesis of the native human programmed death ligand 1 immunoglobulin V-like (PD-L1 IgV) domain,[35] was achieved for the first time by the NBT strategy, demonstrating its potentials in difficult protein synthesis.

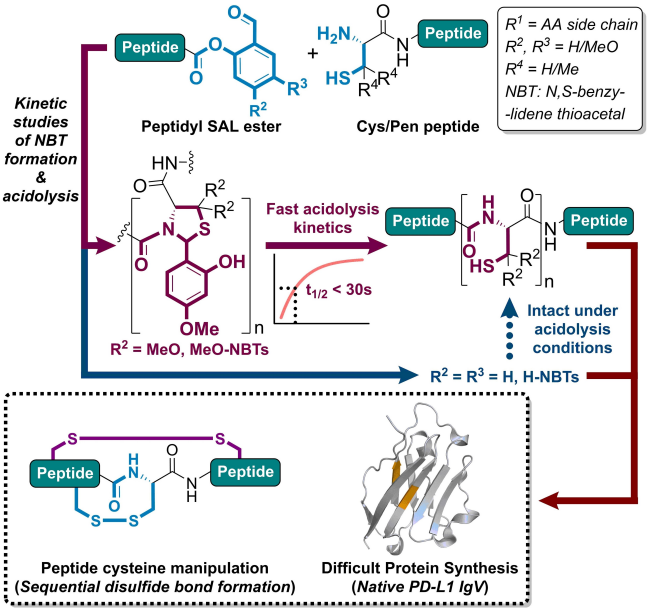


Figure 1. Discovery of the MeO-NBT with fast acidolysis kinetics for applications in peptide manipulation and difficult protein synthesis.

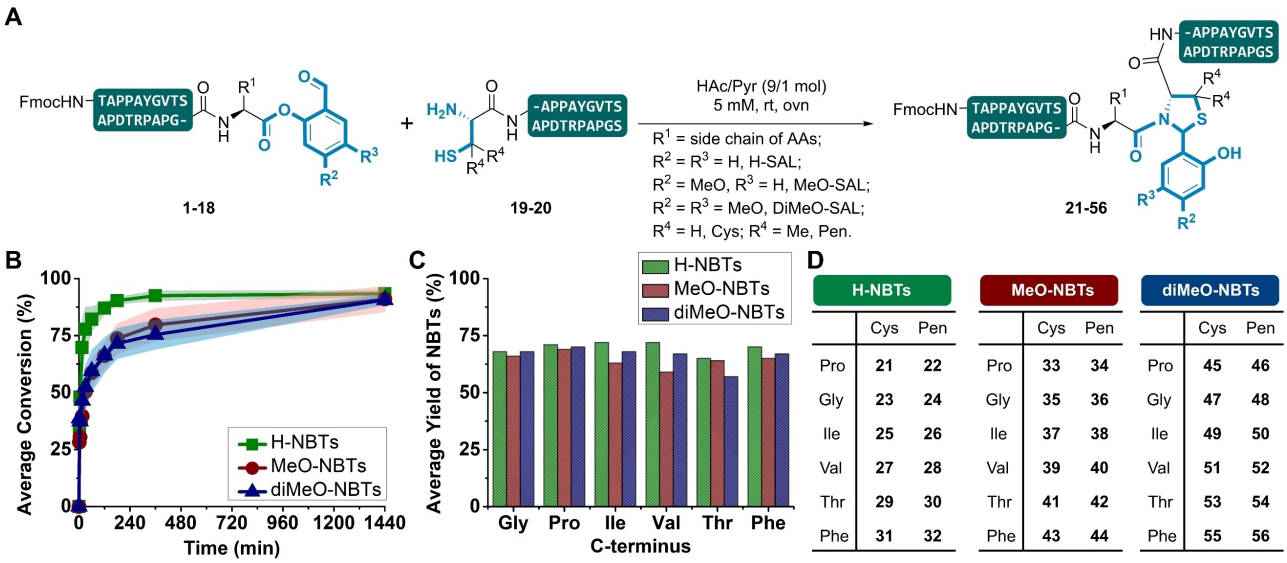
Results and Discussion

Kinetic Studies of NBT Formation by CPL

Before the kinetic investigation of CPL and NBT acidolysis, peptidyl salicylaldehyde esters **1**–**18** and Cys/Pen peptides **19**–**20** were prepared by 9-Fluorenylmethoxy-carbonyl solid-phase peptide synthesis (Fmoc-SPPS) (Figure 2).[24] A 20-mer peptide sequence derived from MUC1 repeating unit was chosen as the model for the formation and acidolysis of NBTs.[36] With variation of steric hindrance, 6 representative amino acid residues (i.e. Gly, Pro, Ile, Val, Phe and Thr) were then selected as the C-terminus of peptide salicylaldehyde esters to evaluate their effects on the following reactions.[37] Thus, native salicylaldehyde (H-SAL), 4-methoxyl salicylaldehyde (MeO-SAL) and 4,5-dimethoxyl salicylaldehyde (diMeO-SAL) were involved to produce different substituted NBT structures. Subsequently under pyridine-acetic acid conditions (HAc/Pyr), all these peptide SAL esters were ligated to peptides with N-terminal Cys (**19**) or Pen (**20**), affording 36 ligation intermediates **21**–**56** in total with mixed-and-match of ligation sites and substituted salicylaldehydes (Figure 2A). Next, the ligation kinetics of above models were studied, and the average results were summarized (Figure 2B, see Supporting Information for more details). When the H-SAL was applied, ligations were smoothly accomplished within few hours and all ligation sites (Xaa-Cys/Pen) tested in this study had a relatively minor effect on the ligation rate even in the case of Ile-Pen or Pro-Pen. By contrast, the addition of one -OMe group to the SAL affected more the ligation kinetics, which was acceptable as the electron-donating *p*-OMe group decreased the condensation reactivity of aldehyde in the peptide SAL ester. Fortunately, MeO-SAL mediated CPLs proceeded to complete after elongating the reaction time to 12 h at all examined Xaa-Cys/Pen. Meanwhile, one extra -OMe group to the MeOSAL continued to slow down the ligation in a minor way, which may be probably due to the less efficient acyl transfer by stabilization of the phenolic ester as well as aldehyde in diMeO-SAL. Nevertheless, comparable isolated yield of ligation intermediates could be obtained in all cases (60–70 %, Figure 2C), providing substrates bearing diverse NBT structures for the next step study.

Acidolysis Kinetics of NBTs

In an initial attempt, we surprisingly found that peptide **34** with MeO-NBT at Pro-Pen site was completely acidolyzed by TFA/TIPS/H_2O in only 15 min, while peptide **22** with H-NBT at the same ligation site remained intact for over 60 min. As reported previously, the acidolysis of H-NBT at the Pro-Pen site was very sluggish and required harsher TFA/TMSOTf/EDT conditions for longer time (2–6 hr). The unexpectedly rapid acidolysis of **34** prompted us to



H-NBTs	Cys	Pen
Pro	21	22
Gly	23	24
Ile	25	26
Val	27	28
Thr	29	30
Phe	31	32

MeO-NBTs	Cys	Pen
Pro	33	34
Gly	35	36
Ile	37	38
Val	39	40
Thr	41	42
Phe	43	44

diMeO-NBTs	Cys	Pen
Pro	45	46
Gly	47	48
Ile	49	50
Val	51	52
Thr	53	54
Phe	55	56

Figure 2. Synthesis of model peptides carrying different substituted NBTs at various ligation sites. A) Synthetic scheme of model peptides. B) Average ligation kinetics of the formation of different substituted NBTs with fade color filling of error bar. C) Average HPLC isolated yield of peptides with NBTs at the linkage of Xaa-Cys/Pen. D) Tables of model peptide NBTs prepared in this study.

investigate the acidolysis of substituted NBTs systematically. Under TFA/TIPS/H_2O conditions, previously obtained ligation intermediates **21–56** with substituted NBTs at Xaa-Cys/Pen sites were subjected to acidolysis where aliquots of the reaction mixture were taken and quenched in second scale timepoints (Figure 3). Brief summary of NBTs acidolysis kinetics was included in average of all ligation sites examined at Figure 3B, and kinetic plots for each individual C-terminal Xaa were showed in Figure 3C–3G (please refer to Supporting Information for more detailed information). Excitingly, peptide **33–56** with MeO- or diMeO- NBT both underwent acidolysis in an even faster way than expected, affording around 75 % conversion already in just the first minute of the reaction. In details, such fast acidolysis of substituted NBTs well proceeded with the pseudo-first order kinetics with relatively large apparent rate constant at the range of $2.4–2.6\times10^{-2}$/s, giving the averaged half-life within only 30 s. According to acidolysis results of NBTs, one methoxy group was found to be crucial to obtain the rapid conversion. The addition of the second MeO- group in diMeO-NBTs did not accelerate the acidolysis to another magnitude and in some cases, it even exhibited a slightly slower kinetics than that with MeO-SAL (e.g. Pro-Pen in Figure 3C, Phe-Pen in Figure 3G). Interestingly, the ligation sites only had a minor effect on the acidolysis of MeO-/diMeO-NBTs. Except for the case of Pro-Cys/Pen, faster acidolysis could be observed with the increasing steric hindrance from Xaa on the NBT structure. For example, over 95 % conversion could be obtained at 120 s for acidolysis of MeO-/diMeO-NBT at Val/Ile-Cys (Figure 3D and 3E), while that at Gly/Thr-Cys site required slightly longer reaction time to ~3 min (Figure 3C and 3F). The difference of acidolysis between them is probably due to the accumulation of strain in the NBT ring when the side chain of the C-terminal ligation site became increasingly bulky. This is also supported by the replacement of the Cys by bulkier Pen at the N-terminal ligation site, which also had a larger apparent constant than that with Cys (See Supporting Information for Figure S3–4.6 and S3–4.7). As for the most challenging Pro-Pen, the NBT backbone modification could be fully removed by elongating the acid treatment to 5–10 min. It should be noted that although there were a pair of NBT isomers generated during the ligation due to the generation of a new stereocenter at the acetal position of the thiazolidine ring, either the major or the minor MeO-/diMeO-NBTs isomer could undergo fast acidolysis (Figure 3H). Furthermore, the generation of MeO- or diMeO-salicylaldehyde was directly observed together with the yellow to brown color of the reaction mixture in the early stage of acidolysis (<60 s), which was then gradually reduced into the related benzyl alcohol by the presence of silane. It might indicate a different acidolysis mechanism of NBTs compared to that with the *p*-methoxybenzyl- (PMB−) or 2-hydroxy-4-methoxybenzyl- (HMB−) protecting group.

While the acidolysis of MeO-/diMeO-NBTs showed impressive kinetic characteristics independent of ligation sites Xaa-Cys/Pen, peptides **21–32** with H-NBT in most of cases were barely affected (1–2 % conversion) in 10 minutes, consisting with previous results. Meanwhile, extending the incubation time of H-NBT containing peptide **25** at Ile-Cys in TFA even up to 18 hr increased little conversion, leaving the starting material as the dominant part of the reaction with some side reactions (Figure 3I). Meanwhile, a slightly higher conversion (~5 %) of H-NBT was detected at Ile-Pen (Figure 3D) and Val-Pen (Figure 3E), probably due to the increased ring strain from steric hindered amino acids as discussed above. In brief, H-NBTs and MeO-/diMeO-NBTs shared a very distinct acidolysis kinetics under TFA/TIPS/H_2O conditions, where the former peptides with H-NBT structure could remain intact and the latter ones could be

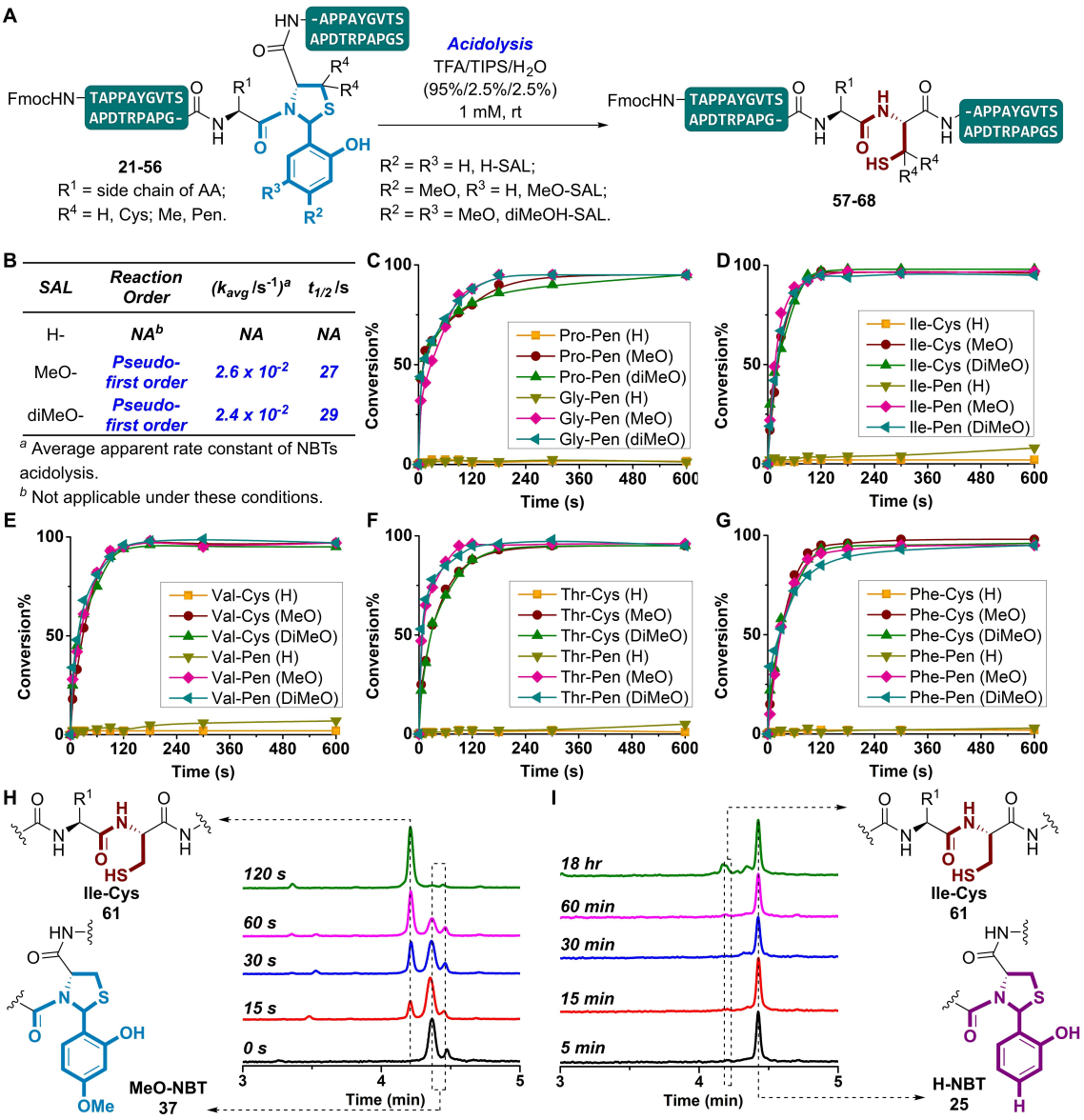


Figure 3. Acidolysis kinetics of NBTs in peptides. A) Peptide acidolysis reaction in this study. B) Table of key kinetic parameters of different substituted NBTs in average of Xaa-Cys/Pen sites. C–G) Acidolysis kinetics of NBTs at different C-terminal ligation sites (e.g. Pro, Gly, Ile, Val, Thr and Phe). H) Comparison of UPLC spectra at different timepoints of MeO-NBT acidolysis at Ile-Cys. I) Comparison of UPLC spectra at different timepoints of H-NBT acidolysis at Ile-Cys.

acidolyzed in few minutes. The difference may also provide a potential kinetic window for flexible manipulation of Cys/Pen protection in peptide or protein chemical synthesis for their differentiation in one simple core structure.

Controllable Acidolysis of NBTs in Peptide Cys Manipulation

After the detailed kinetic studies of peptide NBT acidolysis, we continued to explore the scope and limitations of substituted NBT in peptide synthesis (Figure 4). Considering the comparable ligation efficiency, acidolysis characteristics and wide commercial availability, the MeO-NBT was selected over diMeO-NBT for the following study. In addition to the established solution-phase CPLs to generate one or multiple NBT structures in peptides, NBTs could be also facilely introduced by the on-resin ligation during Fmoc-SPPS. In the latter strategy, the substituted salicylaldehyde esters of Fmoc-protected single amino acids were ligated to the N-terminal free Cys under the pyridine-acetic acid conditions, producing NBTs at the resin-bound protected peptide chain. Similar to the highly twisted *N,O*-benzylidene acetal dipeptides (NBDs) that we previously developed, the insertion of *N,S*-benzylidene thioacetals to the growing peptide chain also potentially facilitated the difficult peptide synthesis by preventing peptide aggregation by blocking its amide bond interaction as well as changing the main chain conformation. After SPPS, the easy removal

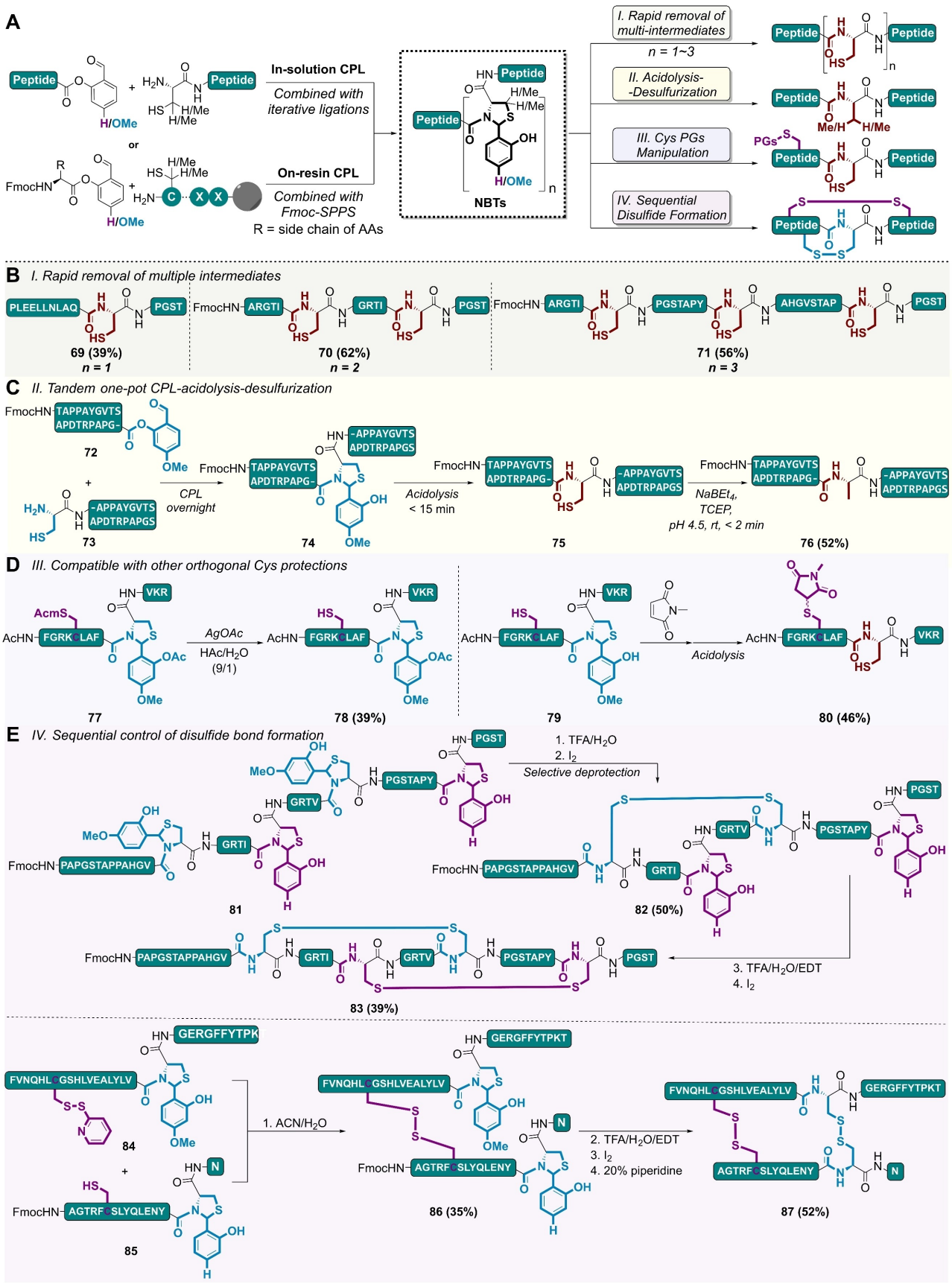


Figure 4. Strategies for the installation of NBTs and their applications in peptide synthesis.

of one or multiple MeO-NBTs during TFA global deprotection offers a practical and robust way to obtain peptides in high purity. Alternatively, the NBTs could be also maintained after SPPS by capping an Ac group to the phenolic group, which changed the electron density in NBTs and made it reluctant to the TFA global deprotection. Several examples of NBTs construction by the on-resin strategy were demonstrated in good yields, covering various types of Xaa-Cys linkages (see Supporting Information for **M1**–**M10**, 18 %–35 % based on resin loading).

To examine the idea of rapid removal of multiple MeO-NBTs, peptides with 1–3 CPL intermediates were prepared accordingly (Figure 4B). Pleasingly, the extra numbers of MeO-NBTs did not compromise the efficiency of acidolysis, and after 15 minutes the completely acidolyzed product **69**–**71** could be purified in high yields. In addition, the fast acidolysis could be tandem with the $NaBEt_4$-mediated superfast desulfurization together to achieve rapid peptide manipulation of ligation-acidolysis-desulfurization (Figure 4C).[29] In this demonstration, peptidyl MeO-SAL ester **72** was firstly ligated with peptide **73** to form peptidyl MeO-NBT **74** after overnight incubation in HAc/Pyr. Then, a 15-min standard MeO-NBT acidolysis was performed followed by cold ether precipitation to remove excess TFA cocktails and redissolving acidolyzed peptide **75** in pH 4.5 citrate buffer containing TCEP. Subsequently, the Cys residue was cleanly desulfurized to Ala in peptide **76** by sodium tetraethylborate in an add-and-done manner, finishing the acidolysis-desulfurization process within 30 min.

Meanwhile, NBT exhibited well compatibility with other widely used Cys protecting groups (Figure 4D).[38] For the first model peptide **77** equipped with both Acm and MeO-NBT protection, the deprotection of Acm group by silver acetate in acetic acid was smooth to produce peptide **78** without affecting the integrity of Ac-capped MeO-NBT at all. The acetyl group was found to be important to stabilize the MeO-NBT during long time treatment of AgOAc in AcOH. To the best of our knowledge, very few Cys protecting groups could tolerate such conditions and at the same time could be readily removed under mild conditions.[39] Normally removing Acm has been applied as the last step during peptide synthesis. Thus, the compatibility of capped MeO-NBT may offer another flexibility of orthogonal manipulation on demand. On the other hand, the free Cys thiol of model peptide **79** with MeO-NBT could be prepared by SPPS and then selectively modified by N-methyl maleimide.[40] In the presence of the thiol-maleimide adduct, the MeO-NBT moiety could be solely detached by acid treatment to release the other Cys thiol of peptide **80** in 46 % isolated yield for other applications.

More importantly, the compatibility and orthogonality of NBT can enable the sequential disulfide bond formation as well to construct complex peptide architecture (Figure 4E). Through iterative Cys/Pen ligations, peptide **81** was prepared with the controllable introduction of two H-NBTs and two MeO-NBTs in between. Under the first TFA treatment for 15 minutes, only MeO-NBTs of **81** were acidolyzed while its H-NBTs remained unchanged by kinetic control as disclosed in Figure 3. Followed by iodine oxidation, the released two Cys thiols from MeO-NBTs specifically formed a pair of disulfide bond, giving the product **82** in 50 % yield over two steps. Next, harsher TFA cleavage conditions containing EDT were applied as the second acid treatment to remove the remaining two H-NBTs in few hours. Later the routine disulfide bond formation was achieved between these two newly released thiols to construct the final product **83** with two disulfide bonds successfully in 39 % HPLC yield. In another example, the first intermolecular disulfide bond was formed between the pyridyl-disulfide activated peptide **84** and peptide **85** with free thiol to generate a heterogenic peptide dimer **86** carrying NBTs at each chain with 35 % yield. Then after TFA/H_2O/EDT treatment to remove H-NBT and MeO-NBT in one-pot, the second interchain disulfide bond was built by iodine oxidation. Deprotection of the Fmoc group by 20 % piperidine afforded peptide **87** with two intermolecular disulfide bridges in 52 % isolated yield.

To sum up, different applications of H-NBT and MeO-NBT have been examined including the fast removal of MeO-NBTs, tandem usage of $NaBEt_4$-desulfurization, Cys protecting group manipulation on demand and sequential disulfide bond formation. With further combination with other orthogonal Cys protecting groups, the NBT system may provide more flexibility in constructing peptides or proteins with complicated structures.

NBTs as Controllable Cys Protecting Groups in PD-L1 Protein Synthesis

After demonstrating utilities of the controllable NBTs acidolysis in peptide synthesis, we continued to explore whether it could be practical as well in chemical protein synthesis, especially for those difficult proteins. Programmed death ligand 1 (PD-L1) is an important and challenging immune-checkpoint protein[41] of our interest. Overexpression of PD-L1 has been found in many types of tumor cells, which can evade immune surveillance by interacting with its receptor the programmed cell death protein 1 (PD1) on the surface of activated T cells, B cells and macrophages.[35] The blockade of such PD-1/PD-L1 interaction by relative antibodies can enhance immune cell activation to eliminate the escaped tumor cells, serving as the next-generation cancer immunotherapy.[42] Many FDA-approved PD-L1 antibodies have been applied for various types of tumors such as melanoma, non-small cell lung cancer and hepatocellular carcinoma.[43] But only 20–40 % of patients could respond to the PD-1/PD-L1 blockade, which is still not clearly understood yet. The chemical synthesis provides precise chemical engineering of the PD-L1 and even can reverse all its chirality into a mirror-image protein, which may help elucidate the reason of primary or acquired resistance of PD-1/PD-L1 blockade as well as developing more potent immunotherapeutic inhibitors.

The crystal structure of PD-1/PD-L1 complex indicates their interaction occurring through their IgV domains.[44] Thus, synthesizing such domain is ideal for further investigation. However, the chemical synthesis of the IgV domain

of an immune checkpoint protein containing two antiparallel beta sheet sandwich structure is difficult due to its serious aggregation and poor solubility from the fragment SPPS to the final ligation assembling. Among strategies developed, adding a non-native poly-arginine or poly-histidine tag with abundant positive charges at the N-/C-terminus of the target protein has been commonly adopted to solve the aggregation and solubility problems. Previously, the chemical synthesis of the *d*-PD-L1 IgV domain with an additional C-terminal poly-Arg tag was developed for bio-panning of potent *d*-peptide inhibitors by phage display, from which a hit peptide was obtained with sub-micromolar PD-L1 binding affinity.[45] We envisioned that through introducing our fast-to-remove and aggregation-preventing NBTs, those synthetic challenges as mentioned above could be tackled to complete the chemical synthesis of native PD-L1 IgV domain.

To establish the synthesis of native PD-L1 IgV domain (17–132), we divided this 116-AA domain into six fragments **88**–**93** to proceed three NCLs, one CPL and one STL (Figure 5). Ala52 of **89**, Ala85 of **90** and Ala121 of **92** were temporarily mutated to Cys for NCL, which could be restored back to native Ala by desulfurization. To differentiate the native Cys residues and mutated ones, orthogonal protection of Cys39 of **88** and Cys114 of **91** by substituted NBTs were introduced by the on-resin method and in-solution CPL respectively. After the desulfurization of mutated Cys residues, rapid acidolysis would be carried out to remove two NBTs for the final disulfide bond formation during protein refolding. Accordingly, peptidyl hydrazide **88** and **89**, peptidyl SAL ester **90** and **92** and fragment **93** were prepared by Fmoc-SPPS. As for fragment **90**, an isoacyl dipeptide incorporated at Ile101-Thr102 was necessary to disrupt the secondary interaction in the peptide chain, facilitating its purification. In another fragment **91**, an uncommon trifluoroethanol (TFE) ester was adopted as it could prevent the non-productive hydrazone formation between peptidyl hydrazide and peptidyl SAL ester during CPL. Meanwhile, the peptidyl TFE ester could be post manipulated to a peptidyl hydrazide by hydrazine treatment so that it could be still activated for the ligation thereafter.

With all starting materials in hand, we performed the first NCL through $NaNO_2$ activation[46] between MeO-NBT containing peptidyl hydrazide **88** and fragment **89** to obtain the ligated peptide **94** in 26 % yield. Then, peptide **90** with C-terminal 4-(2-propynyloxy)salicylaldehyde ester was ligated with the N-terminal Cys of peptide **91**. Although the isopeptide linkage in **90** was unstable in HAc/Pyr conditions and would gradually undergo acyl transfer to a native amide linkage, the generation of the alkoxy-NBT at the same time could serve as another aggregation disruptor to maintain the good solubility of the ligation intermediate, finishing a "backbone interference relay".[23] Followed by a Cu-catalyzed azide-alkyne click reaction (CuAAC), fragment **95** was obtained in 20 % yield over two steps with a poly-Lys solubilizing tag linked to the alkoxy NBT through a triazole. Next, the second NCL was smoothly performed to join the activated peptidyl hydrazide **94** and peptide **95** together. Treating the ligated product with aqueous hydrazine in one pot transformed it into peptidyl hydrazide **97** in 48 % isolated yield.

Next, there was only one part left to accomplish the assembling, the PD-L1 C-terminal part (121–132). It was regarded as the most challenging sequence during the PD-L1 synthesis due to its hydrophobicity and tendency of aggregation. In fact, the direct synthesis of it was attempted but the result was very poor because of its serious truncation and extremely poor solubility. Applying our recently developed *N,O*-benzylidene acetal dipeptides (NBDs)-SPPS strategy could efficiently disrupt residue aggregation and suppress the residue truncation to provide much improved quality of the peptide.[48] However, it did not solve the purification problem as the acid-labile NBA could not maintain in the fragment after the global deprotection of Fmoc-SPPS. Repetitive separating the PD-L1 (121–132) resulted in miserable isolated yield and its subsequent ligation was not smooth. To solve the problem, we disconnected it into two fragments **92** and **93** for the installation of ligation intermediate in unprotected peptides, as ligation embedding aggregation disruptor (LEAD).[23] Luckily, the insertion of a *N,O*-benzylidene acetal (NBA) at Thr127, through STL between peptidyl SAL ester **92** and peptide **93** was successfully. Later, removal of N-terminal Thz group by methoxyamine successfully afforded the PD-L1 (121–132) fragment **96** in 29 % HPLC yield. The generated STL intermediate peptide had significantly less aggregation and better solubility, enabling the smooth purification and success of the following ligation.

Finally, the third NCL of peptide **96** and peptide **97** was performed to assemble the full length of PD-L1 IgV domain with Ala mutation and CPL/STL ligation intermediates in 43 % yield. It should be mentioned that the presence of the alkoxy-NBT with a polyLys tag at Cys98 was critical to provide sufficient solubility of the product in the subsequent desulfurization. Otherwise, the substrate would be totally insoluble even in the 8 M saturated guanidine buffer, leading to messy desulfurization. By contrast, completely desulfurized **98** was obtained in 43 % HPLC isolated yield with the help of NBTs and LEAD. Such difference indicates that even though the CPL/STL intermediate breaks down the aggregation, charges from solubilizing tag may still be required to balance the over hydrophobicity of the peptide. Subsequently, one-pot acidolysis of **98** to remove *N,O*-/*N,S*-benzylidene (thiol)acetals proceeded, affording the unfolded **99** in 35 % yield. Furthermore, **99** was subjected to folding conditions to produce the folded PD-L1 IgV (17–132) **100** in 25 % yield. Compared to the UPLC-MS spectra before and after folding of PD-L1, a significant shift of retention time in the UPLC trace as well as changes of MS pattern suggested the formation of high-dimension protein structure and formation of the Cys39-Cys114 disulfide bond, which was also supported by deconvoluted MS spectra showing 2-Da difference (Figure 5B–5D, see Supporting Information for more details). Meanwhile, the highly abundant beta-sheet of **100** was characterized by circular dichroism (CD) spectra, exhibiting similar structure as that of the recombinant PD-L1 IgV with a C-terminal His tag (Figure 5E).

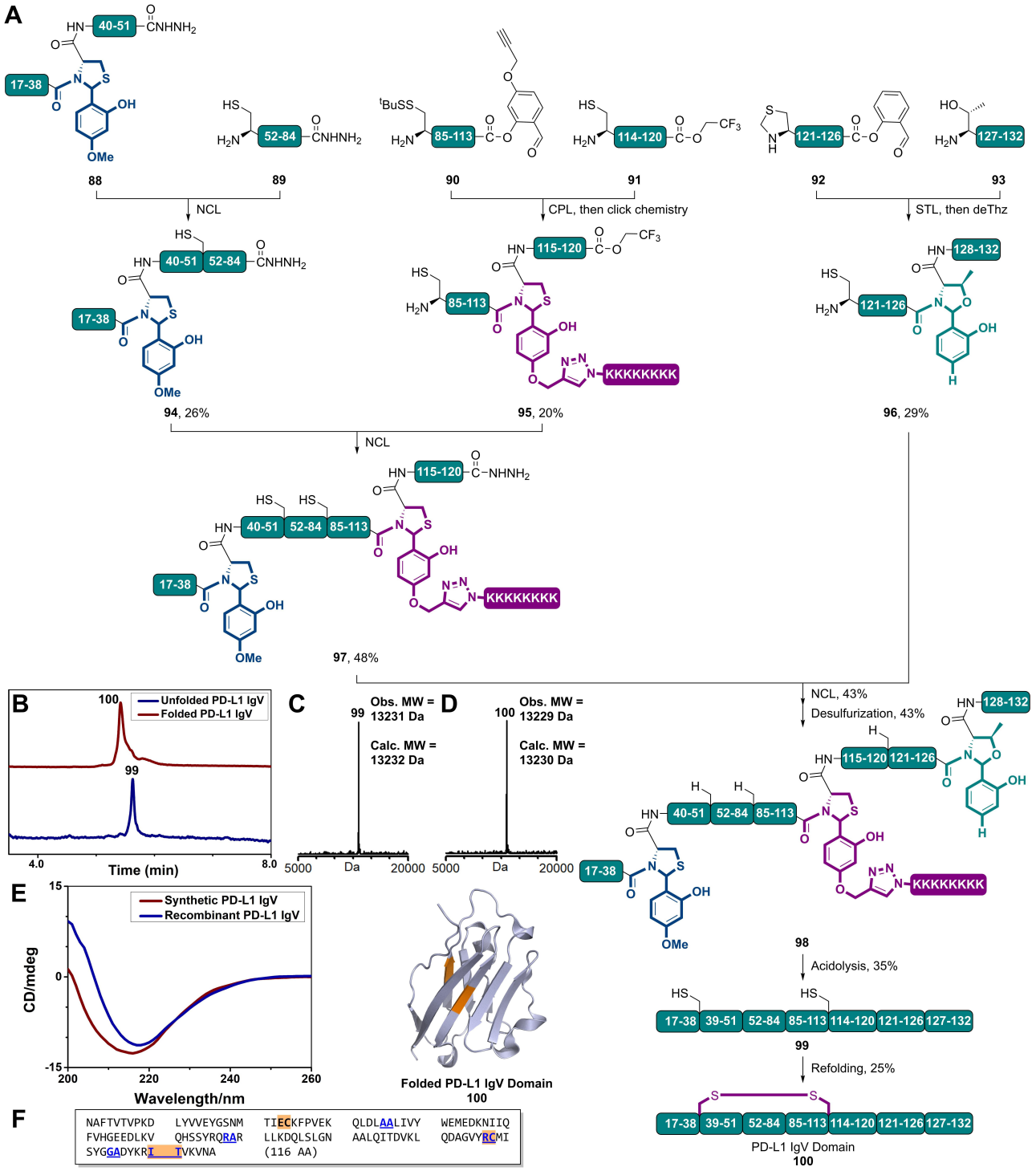


Figure 5. Application of NBT for the chemical synthesis of the native PD-L1 IgV domain. A) Synthesis of **100**. B) UPLC spectra of PD-L1 before and after folding. C) Deconvolution MS spectrum of unfolded PD-L1 **99**. D) Deconvolution MS spectrum of folded PD-L1 **100**. E) Comparison of CD spectra of synthetic PD-L1 IgV and recombinant one. F) Sequence of native PD-L1 IgV domain with NBT sites highlighted and ligation sites underlined.

In summary, the first chemical synthesis of native PD-L1 IgV domain (17–132) **100** has been successfully achieved by the NBT strategy which minimizes fragment aggregation, increases solubility and allows for easy removal.

Conclusion

Through detailed study of both the formation and acidolysis of *N,S*-benzylidene thioacetals in peptides, MeO-NBTs showed remarkable acidolysis kinetics regardless of the

amino acid linkage at the only cost of an acceptably slower formation than that of H-NBTs with comparable yields. The ready removal, controllable release over H-NBTs, and wide tolerance toward other protecting group techniques made MeO-NBTs a flexible and convenient tool for peptide synthesis, including fast acidolysis of multiple NBTs, sequential disulfide bond formation and tandem applications with other chemistry. More importantly, the intrinsic nature of aggregation interference and facile attachment of other helpful auxiliaries in NBT are advantageous for the construction of highly difficult proteins as demonstrated by the first chemical synthesis of the native PD-L1 IgV domain. It is believed that the synthesis of other important but challenging proteins could be robustly built up by combining the NBT strategy with ligation chemistry, providing a foundation for fundamental chemical biology investigations and novel therapeutic development.

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Conflict of Interest

The authors declare no conflict of interest.

Data Availability Statement

The data that support the findings of this study are available in the supplementary material of this article.

Keywords: N,S-Benzylidene Thioacetals · Kinetics · Peptide Ligation · Peptide Manipulation · Chemical Protein Synthesis

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