On-demand Detachment of Maleimide Derivatives on Cysteine to Facilitate (Semi)Synthesis of Challenging Proteins

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Abstract

The maleimide group is a widely used reagent for bio-conjugation of peptides, proteins and oligonucleotides employing Michael addition and Diels-Alder cycloaddition reactions. However, the utility of this functionality in chemical synthesis of peptides and proteins remains unexplored. We report, for the first time that Pd^{II} complexes can mediate the efficient removal of various maleimide derivatives in aqueous conditions. Maleimide removal by Pd^{II} was applied for the synthesis of two ubiquitin activity-based probes (Ub-ABPs) employing solid phase chemical ligation (SPCL). SPCL was achieved through a sequential three segments ligation on a polymer support *via* a maleimide anchor. The obtained probes successfully formed the expected covalent complexes with deubiquitinating enzymes (DUBs) USP2 and USP7, highlighting the use of our new method for efficient preparation of unique synthetic proteins. Importantly, we demonstrate the advantages of our newly developed method for the protection and deprotection of native cysteine with a maleimide group in a peptide fragment derived from thioredoxin-1 (Trx-1) obtained *via* intein based expression to enable ligation/desulfurization and subsequent disulfide bond formation in a one-pot process.

Introduction

Maleimide is a ubiquitous functionality often used for bio-conjugation of peptides, proteins and oligonucleotides due to its relative high stability, selectivity and electrophilic properties.[1] Specifically, maleimide is employed as a Michael acceptor for conjugating thiols or dienophiles for Diels-Alder cycloadditions. Maleimide chemistry allows the production of fluorescently labeled proteins,[2] antibody-drug conjugates,[3] PEGylated proteins,[4] protein-DNA hybrids,[5] and cyclic peptides among other complex conjuagtes.[6] Despite its wide application, there are still some limitations regarding the stability of maleimide based linkers. Maleimides are susceptible to the retro-Michael reaction, which cleaves the thioether linkage under a reducing environment, leading to the loss of cargo.[7] Therefore, most of the efforts have been focused on preventing the retro-Michael reaction in order to stabilize the maleimide conjugates.[8] Another disadvantage is that some antibody-drug conjugates connected through cysteine-maleimide chemistry require additional linkers that can be cleaved either enzymatically[9] or chemically[10] to release the payload in tumor sites. Developing on demand detachment of the maleimide linkage has potential for expanding the utility of maleimide based linkers for antibody-drug conjugates and for chemical proteomics.[11] In addition, such an approach might find useful applications in peptide and protein syntheses employing the maleimide as a reversible protecting group and as a cleavable linker.

Chemical protein synthesis permits unlimited site specific modifications of the protein target such as the introduction of posttranslational modifications (PTMs), D-amino acids, affinity and fluorophore tags.[12] However, often the selection of protecting groups (PGs)

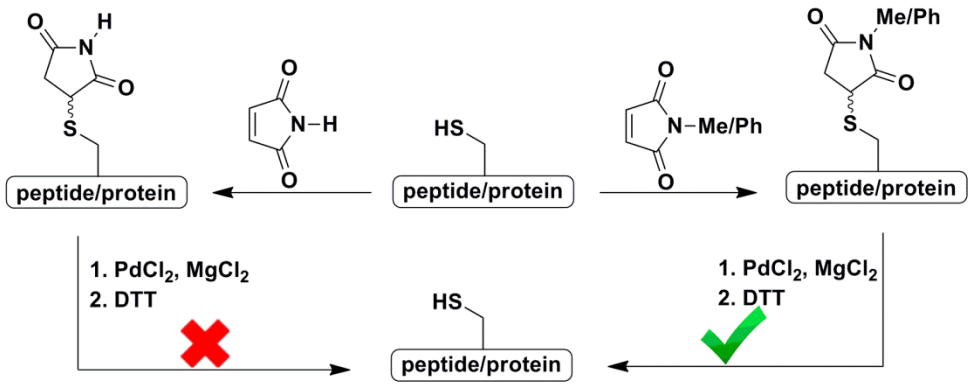
is highly crucial for achieving successful synthesis of the target protein.[13] In recent years our group has made tremendous efforts to expand this chemical toolbox, predominantly by developing palladium mediated efficient cleavage of several PGs to facilitate chemical peptide and protein synthesis.[14] These PGs have enabled sequential ligation,[15] selective desulfurization, and one-pot disulfide bond formation.[16] This chemistry was also extended for introducing solubilizing tags[17] and new linkers[18] for *in vitro* and cellular applications. Despite their importance, the requirement for pre-installation of these PGs during synthesis of peptide segments (using SPPS), limits their applicability in particular when dealing with recombinant peptide fragments. Therefore, the development of reagents which enable on demand attachment/detachment of free Cys residues under aqueous conditions is of great interest. Despite some progress in this direction the current methods have limitations. For example, the phenacyl group was used as a Cys PG for expressed fragments to enable protein semisynthesis.[19] Similarly, the trityl group was used as Cys PG to facilitate disulfide bond formations.[20] However, the instability of phenacyl group when using methoxy amine during Thz opening,[19b] and the instability of the trityl PG during desulfurization makes these methods less attractive.[19c] In addition, both methods require organic solvents for either the protection or deprotection step, which have the potential to affect peptide solubility and makes such an approach incompatible with one-pot synthesis. Therefore, the development of more efficient reagents, specifically those that are stable under native chemical ligation (NCL)/desulfurization conditions and compatible with one-pot deprotection/disulfide bond formation for protein (semi)synthesis would have useful applications.

The structural resemblances between the acetamidomethyl (Acm) and maleimide PGs, inspired us to investigate the cleavage reaction of the latter PG using palladium chemistry. Herein, we demonstrate for the first time that Pd^{II} complexes mediate the efficient cleavage of maleimide PG on Cys under aqueous conditions. We further show the use of maleimide as a stable linker during solid phase chemical ligation (SPCL) for successful synthesis of ubiquitin activity-based probes (Ub-ABPs) for selective labeling of two different deubiquitinating enzymes (DUBs). In addition, we applied our strategy for the protection and deprotection of native Cys side chains in a peptide fragment obtained *via* intein based expression to enable the semisynthesis of the thioredoxin-1 (Trx-1) protein by means of ligation/desulfurization and disulfide bond formation in one-pot reaction.

Results and Discussion

To examine the potential of maleimide as a PG, we prepared a model peptide **1** (LYRAGC(*N*-Me.Mal)LYRAG), where Cys was masked with the *N*-methyl maleimide group (*N*-Me.Mal), (Scheme 1). This peptide was initially treated with 10 equiv of $PdCl_2$ in 6 M Gn·HCl, pH 7.3 at 37 ^{o}C. Gratifyingly, the reaction furnished the unprotected peptide with a complete conversion in about 4 h (Figure S2B). Encouraged by this result, we examined the influence of other metal complexes on the maleimide decaging reaction. However, none of the examined metal complexes (AuCl, K_2PtCl_6, $NiCl_2$, $CuCl_2$, and $FeCl_3$) was suitable, highlighting the specific role of Pd complex in this reaction (Figure S3B). We then turned our attention to find a better Pd complex, testing different complexes such as $[Pd(allyl)Cl]_2$, $Pd_2(dba)_3$, $Pd(PPh_3)_4$, $Pd(OAc)_2$, Cl_4Na_2Pd, and $PdCl_2$ which among them gave the best results (Figure S4B). Aiming to improve the reaction

efficiency, we investigated the effect of additives for improving the kinetics of the reaction. When 50 equiv of $MgCl_2$ was added along with 10 equiv of $PdCl_2$ in 6 M Gn·HCl, pH 7.3 at 37 ºC, the reaction was drastically improved and produced quantitatively the decaged peptide within 45 min (Figure S5B). The addition of excess $MgCl_2$ could play various roles including preventing the non-productive chelation of the palladium to the side chain reactive functional groups[21, 15d] as well affecting the chemistry of Pd by possibly forming *in situ* $[PdCl_4]^{2-}$.[22] Using additional additives such as 4-mercaptophenylacetic acid (MPAA), tris(2-carboxyethyl)phosphine (TCEP), GSH, $NaHSO_3$ and $Na_2S_2O_5$ did not improve the reaction (Figure S5B). Optimization of the reaction buffer indicated that 6 M Gn·HCl facilitated the fastest reaction (Figure S6B), an observation we have also made in previous systems.[15d] We therefore concluded that the use of 10 equiv of $PdCl_2$, 50 equiv of $MgCl_2$ in 6 M Gn·HCl, pH 5.5 (pH reduction after addition of the $MgCl_2$) at 37 ºC affords the best results for this reaction.



Scheme 1. Schematic representation of installation and removal of maleimide PGs on Cys in peptides and proteins.

Under our established conditions, we examined the feasibility for the cleavage of additional maleimide derivatives such as the *N*-phenyl substituted maleimide. We

therefore prepared the model peptide **2** (LYRAGC(*N*-Phe.Mal)LYRAG) and examined its behavior under our optimized conditions (Figure S9B). The decaged peptide was obtained with a complete conversion within 45 min, similarly to peptide **1**. In contrast, the unsubstituted maleimide in model peptide **3** (LYRAGC(Mal)LYRAG) was completely stable under Pd cleavage conditions (Figure S9C), which clearly emphasizes the requirement of the phenyl or methyl substitution on the imide group to facilitate cleavage by Pd.

In order to gain insight into the reaction mechanism, we synthesized model peptide **4** (LYRAGC(Mal.LYRAG)LYRAG), where the maleimide group was connected with a short peptide (Figure 1A). Peptide **4** was subjected to our cleavage conditions to give two peptide segments; LYRAGCLYRAG and Mal.LYRAG-$[PdCl_4]^{2-}$ complex. Previously it has been show that the abstraction of the acidic hydrogen from the sulfhydryl side chain of Cys (S^{t}Bu), Cys(Acm), and Cys (Trt) leads to β-elimination to cleave the thiol bond.[23] In a different study, it has been shown that Pd is able to coordinate the free imide in the succinimide based derivative and generate a palladium catalyst for a cross coupling reaction.[24] Based on this information and our results, we propose a plausible mechanism for the cleavage of the maleimide group, as depicted in Figure 1D. In case of *N*-substituted maleimide, the chelation of the Pd complex between the thiol and carbonyl groups trigger β-elimination to cleave the C-S bond and form the thiol-Pd and maleimide-Pd complexes, respectively. The thiol-Pd complex, upon treatment with DTT liberates the free thiol. In case of the unsubstituted maleimide, the free imide group is susceptible to chelation with the Pd to form an unproductive complex.

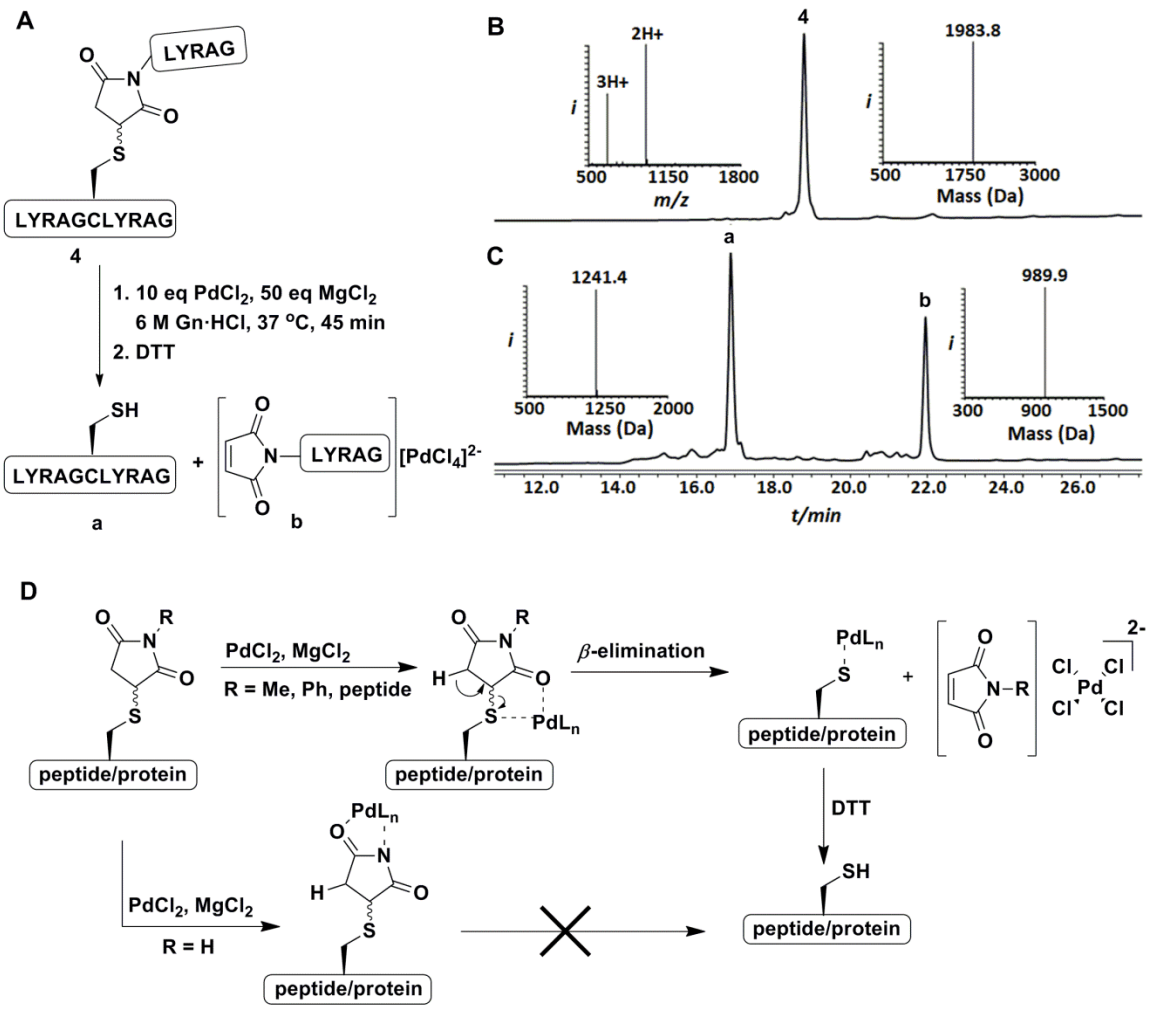


Figure 1. (A) Cleavage of maleimide PG from peptide **4** under optimized conditions. (B) HPLC-MS analysis of the purified model peptide **4** with an observed mass 1983.8 ± 0.1 Da (calcd 1984.3 Da, average isotopes). (C) Cleavage of model peptide **4** under optimized conditions. Peak **a** corresponds to the decaged fragment (LYRAGCLYRAG) with an observed mass 1241.4 ± 0.1 Da (calcd 1242.5 Da, average isotopes). Peak **b** corresponds to the complex of $[PdCl_4]^{2-}$ with (Mal.LYRAG) fragment with an observed mass 989.9 ± 0.1 Da (calcd 990.1 Da, average isotopes). (D) A proposed mechanism for the cleavage of the maleimide group.

Palladium mediated orthogonal cleavage of Thiazolidine and Maleimide

In sequential NCL, masking of the *N*-terminal Cys residue of a peptide fragment by thiazolidine (Thz) is highly desirable to avoid peptide cyclization or polymerization. The cleavage of Thz serving as a PG or linker was reported by our group under $[Pd(allyl)Cl]_2$ and GSH conditions.[18] To examine the orthogonality between the Thz and the maleimide PGs, we synthesized model peptide **5** (Thz-LYRAGC(*N*-Me.Mal)LYRAG), bearing a *N*-terminal Thz and methyl maleimide PGs, (Figure 2A). We then treated this peptide with $[Pd(allyl)Cl]_2$ and GSH in 6 M Gn·HCl, pH 7.3 at 37 ^{o}C. After 45 min, we observed the full conversion of the Thz to the free *N*-terminal Cys, while keeping the maleimide PG completely intact (Figure 2B). However, when model peptide **5** was treated under the optimized conditions, we observed unmasking of the maleimide where the Thz remained stable (88%) along with traces of product having both the Thz and maleimide unmasking (12%) (Figure 2C). This example demonstrates the influence of the specific Pd complex and additive in achieving selective unmasking of two different PGs under aqueous conditions.

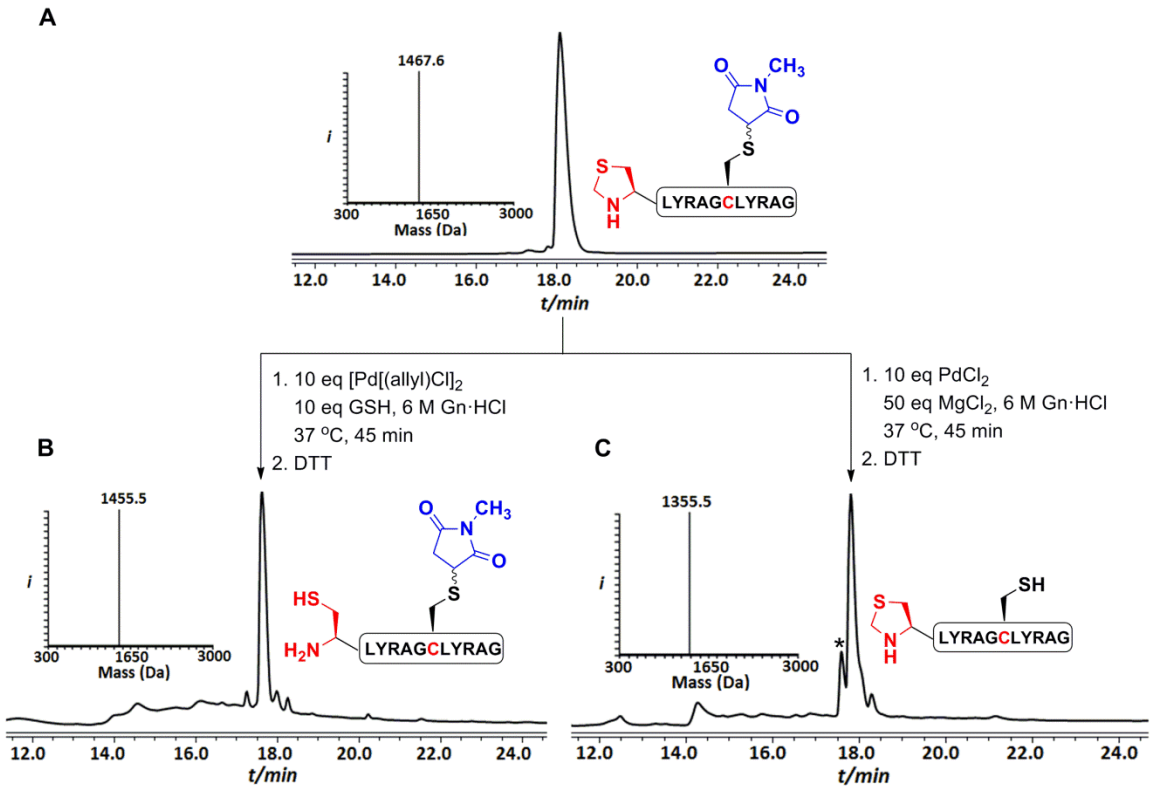


Figure 2. Pd-mediated orthogonal cleavage between the Thz and the maleimide PGs. HPLC-MS analysis: (A) Purified model peptide **5** with an observed mass 1467.6 ± 0.1 Da (calcd 1467.7 Da, average isotopes). (B) Crude product after Thz deprotection with an observed mass 1455.5 ± 0.1 Da (calcd 1455.6 Da, average isotopes). (C) Crude product after the maleimide deprotection with an observed mass 1355.5 ± 0.1 Da (calcd 1356.6 Da, average isotopes). * corresponds to the product with the deprotection of both Thz and maleimide PGs.

The maleimide linker facilitates SPCL

To date, the majority of ligation schemes in chemical protein synthesis have been conducted in aqueous solutions.[12a, 25] However, the shortcomings like handling losses, multiple HPLC purifications, and lyophilization steps prolong synthesis time and limit

the construction of large proteins. Hence, several groups including ours have reported different linkers to enable NCL/desulfurization on the solid support.[26] The success of SPCL relies on the choice of the polymer support, the linker used, NCL/desulfurization and cleavage conditions.

Having established conditions for the selective conversion of the Thz to Cys against the maleimide PG in model peptide **5**, we wondered whether the maleimide could be employed to generate a suitable linker for SPCL (Figure 3). We chose the known PEGA resin as a solid support due to its high swelling properties and compatibility in aqueous buffers.[27] Initially, the maleimide linker was attached to the PEGA polymer support through a spacer of two alanine residues. To enable SPCL for a model system, three peptide segments **6** (Thz-LYRAGLYRAG-Cys), **7** (Thz-LYRAGLYRAG-Nbz (Nbz is *N*-acylbenzimidazolinone),[28] and **8** (LYRAGCLYRAG-Nbz) were prepared by standard Fmoc-SPPS (Figure S12). In the first step, peptide segment **6** was immobilized to the maleimide bearing solid support *via* a covalent bond within minutes and subsequently treated with $Pd[(allyl)Cl]_2$ and GSH to convert the Thz to the *N*-terminal free Cys. After quenching the polymer support with DTT, the first ligation was performed with peptide **7** in presence of MPAA and TCEP for 5 h. Again, the solid support was treated with $[Pd(allyl)Cl]_2$ and GSH to give the *N*-terminal free Cys for the subsequent ligation with peptide segment **8**. In the final step, the solid support was subjected to maleimide cleavage to liberate polypeptide **9**. The entire process took ~13 h for the 6 steps and resulted in 46% isolated yield of product.

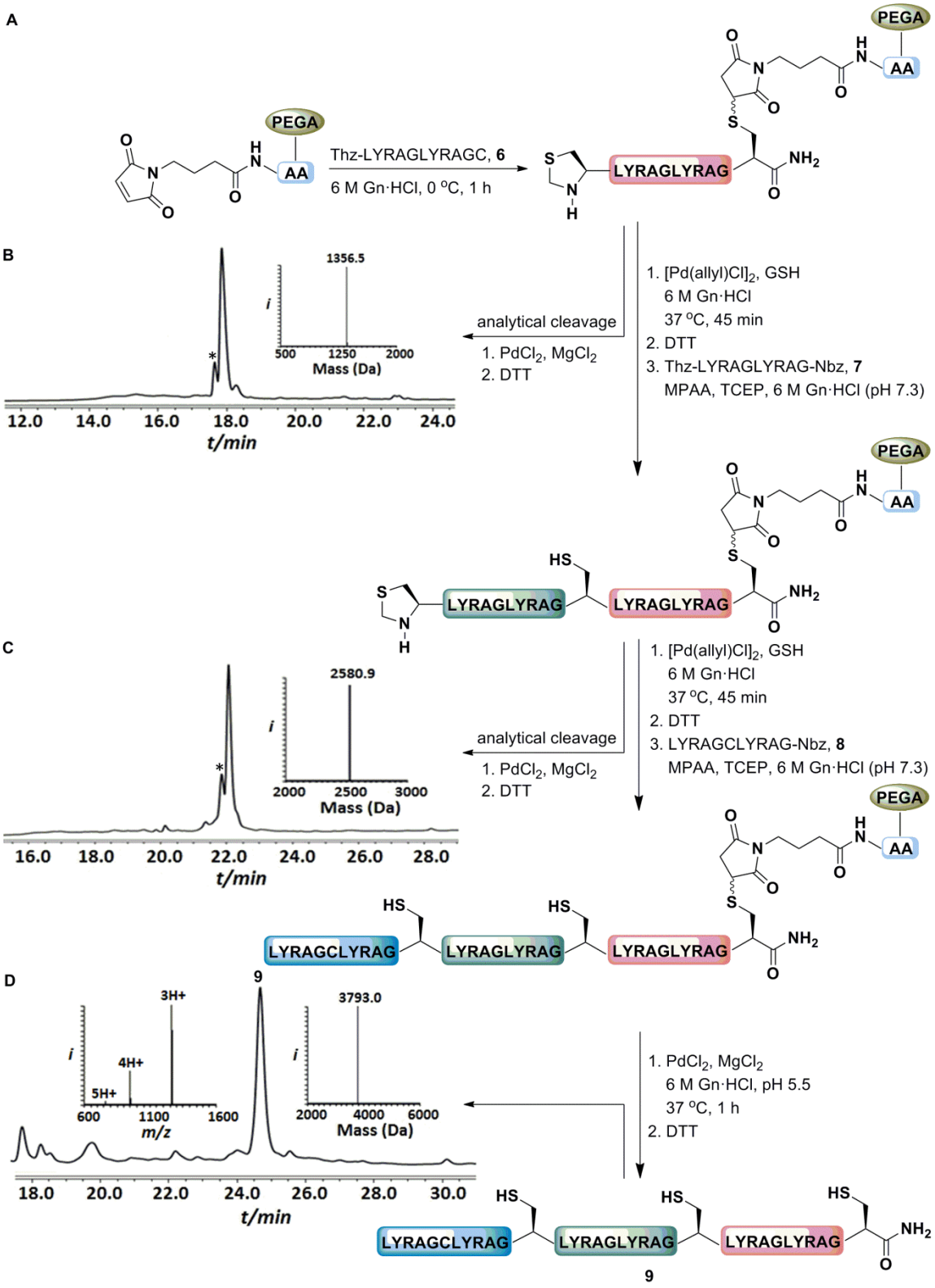

A
PEGA
AA
Thz-LYRAGLYRAGC, 6
6 M Gn·HCl, 0 °C, 1 h
LYRAGLYRAG
NH2
B
1356.5
i
Mass (Da)
t/min
analytical cleavage
1. PdCl2, MgCl2
2. DTT
1. [Pd(allyl)Cl]2, GSH
6 M Gn·HCl
37 °C, 45 min
2. DTT
3. Thz-LYRAGLYRAG-Nbz, 7
MPAA, TCEP, 6 M Gn·HCl (pH 7.3)
HS
LYRAGLYRAG
C
2580.9
3. LYRAGCLYRAG-Nbz, 8
LYRAGCLYRAG
D
9
3H+
4H+
5H+
m/z
3793.0
1. PdCl2, MgCl2
6 M Gn·HCl, pH 5.5
37 °C, 1 h
2. DTT

Figure 3. Overview of our SPCL strategy employing maleimide linker (A) The ligation strategy employing immobilization, elongation and release of the peptide from the solid support. HPLC-MS analysis: (B) Crude product after immobilization with an observed mass of 1356.5 ± 0.1 Da (calcd 1356.6 Da, average isotopes). * corresponds to product of Thz deprotection with an observed mass of 1344.5 ± 0.1 Da (calcd 1344.6 Da, average isotopes). (C) Crude product after first ligation with an observed mass of 2580.9 ± 0.1 Da (calcd 2581.1 Da, average isotopes). * corresponds to product of Thz deprotection with the observed mass of 2568.7 ± 0.1 Da (calcd 2569.1 Da, average isotopes). (D) Crude product after second ligation with an observed mass of 3793.0 ± 0.1 Da (calcd 3794.5 Da, average isotopes).

SPCL employing maleimide linker for the synthesis of (Ub-ABPs):

DUBs are known to cleave the isopeptide bond between the C-terminal glycine residue of Ub and the ubiquitinated proteins. DUBs play crucial roles in the ubiquitination machinery and are emerging as attractive drug targets.[29] Therefore, various Ub-ABPs have been developed to study the role of DUBs in health and disease.[30] Our lab developed a new strategy for the synthesis of Lys-48 and Lys-63 linked di-Ub probes to label different DUBs.[31] Similarly, we also reported the synthesis of ubiquitinated α-globin probe[32] and ubiquitinated H2A probe[15d] for labeling USP15 and Calypso/ASX, respectively. Recently, Champak and coworkers reported the semi synthesis of Ub-dehydroalanine (DHA) probe by using selenocysteine as a latent bio-orthogonal electrophile to capture the TRIM-25-associated DUB, ubiquitin-specific protease 15 (USP15).[33] The Ovaa group demonstrated that Ub-DHA can be used to capture E1-E2-E3 Ub enzymes.[34]

Encouraged by the success of the SPCL in producing model system **9**, we sought to apply these conditions for a more complex system such as Ub-DHA, **14** (Figure 4). Our design for the construction of **14** by SPCL is shown in Figure 4. The sequence of Ub was divided into three segments, including **10** (Thz-Ub(47-75)-Cys), **11** (Thz-Ub(29-45)-Nbz), and **12** (TAMRA-PEG-Ub(1-27)-Nbz), where a fluorophore 5-carboxytetramethylrhodamine (TAMRA) was installed at the *N*-terminal to monitor the DUB capture by using a fluorescent gel (Figure S13). Ala 46 and 28 were temporally replaced with the Thz linkage to enable sequential SPCL. Gly 76 was temporarily mutated to Cys to facilitate the immobilization and late stage DHA formation. With the required segments in hand, the assembly of the polypeptide began with immobilization of peptide segment **10** followed by Thz conversion to the *N*-terminal free Cys. The first ligation was performed with peptide **11** in the presence of MPAA and TCEP for 8 h and subsequently the Thz linkage was opened. Similarly, the second ligation was repeated with peptide **12** for 8 h and subsequently subjected to desulfurization conditions. After the desulfurization step, the solid support was treated under maleimide deprotection conditions to liberate polypeptide **13**, TAMRA-PEG-UbG76C, which was isolated in 37% overall yield from the 7 steps in under 28 h. Later, the purified polypeptide **13** was treated with bisamide[35] to facilitate the formation of Ub-DHA, **14** in 49% isolated yield. Following the same protocol, the assembly of Ubv2.3-DHA probe **17** was obtained in 51% yield (Figure S16). Ubv2.3 is an engineered Ub variant, known to selectively inhibit USP2, which has C-terminal in close proximity to the catalytic active site.[36]

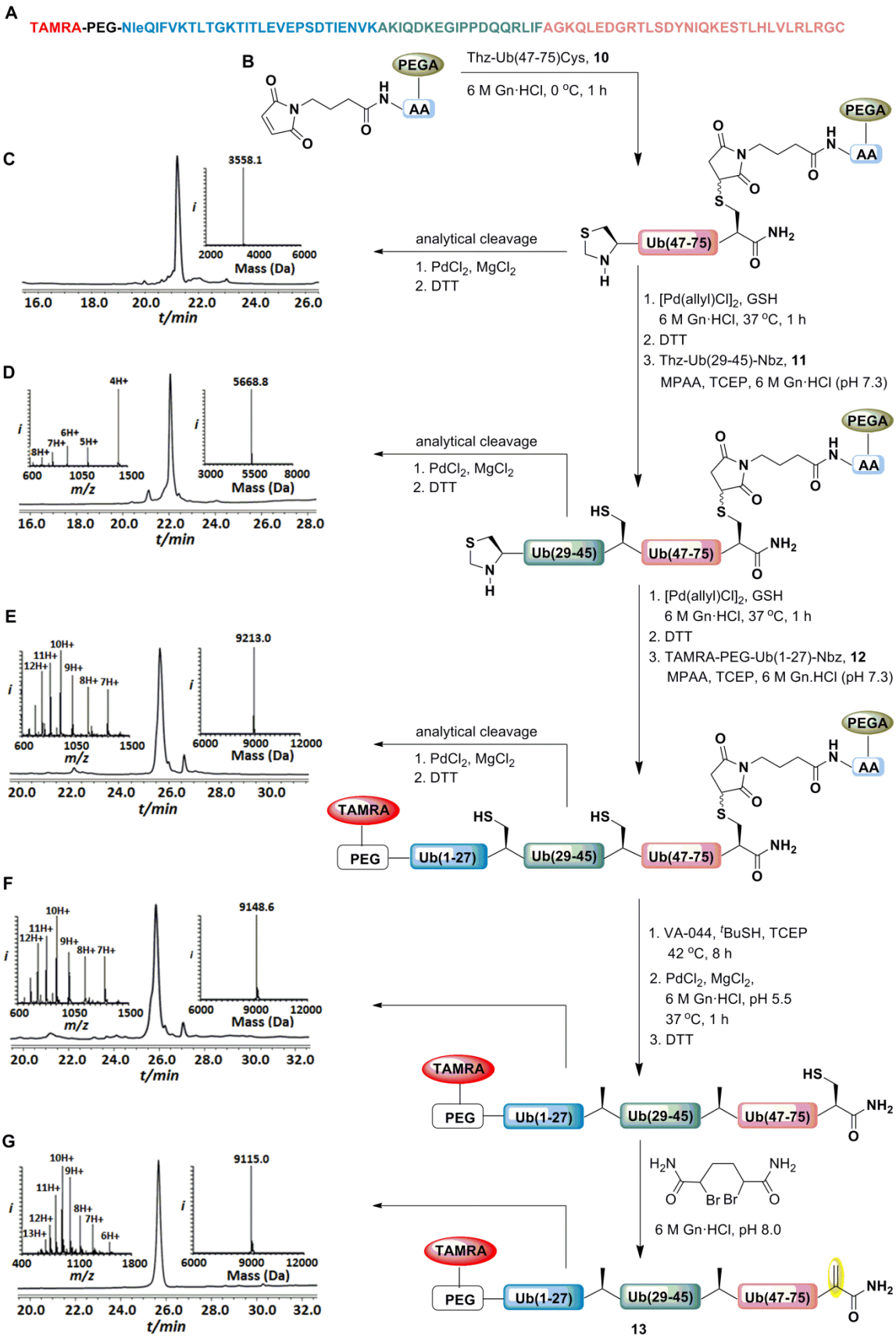

A
TAMRA-PEG-NleQIFVKTLTGKTITLEVEPSDTIENVKAKIQDKEGIPPDQQRLIFAGKQLEDGRTLSDYNIQKESTLHLVLRLRGC
B
PEGA
AA
Thz-Ub(47-75)Cys, 10
6 M Gn·HCl, 0 °C, 1 h
C
3558.1
Mass (Da)
t/min
analytical cleavage
1. PdCl2, MgCl2
2. DTT
Ub(47-75)
1. [Pd(allyl)Cl]2, GSH
6 M Gn·HCl, 37 °C, 1 h
2. DTT
3. Thz-Ub(29-45)-Nbz, 11
MPAA, TCEP, 6 M Gn·HCl (pH 7.3)
D
5668.8
Mass (Da)
t/min
Ub(29-45)
1. [Pd(allyl)Cl]2, GSH
6 M Gn·HCl, 37 °C, 1 h
2. DTT
3. TAMRA-PEG-Ub(1-27)-Nbz, 12
MPAA, TCEP, 6 M Gn.HCl (pH 7.3)
E
9213.0
Mass (Da)
t/min
TAMRA
PEG
Ub(1-27)
1. VA-044, tBuSH, TCEP
42 °C, 8 h
2. PdCl2, MgCl2,
6 M Gn·HCl, pH 5.5
37 °C, 1 h
3. DTT
F
9148.6
Mass (Da)
t/min
6 M Gn·HCl, pH 8.0
G
9115.0
Mass (Da)
t/min
13

Figure 4. Maleimide group facilitates the SPCL strategy for assembling Ub-DHA probe **14**. (A) The sequence of Ub highlighting the ligation sites and different fragments. (B) The ligation strategy employing immobilization, NCL, desulfurization and release of the polypeptide from the solid support and Ub-DHA formation. HPLC-MS analysis: (C) Crude product after immobilization with an observed mass of 3558.1 ± 0.1 Da (calcd 3558.2 Da, average isotopes). (D) Crude product after first ligation with an observed mass of 5668.8 ± 0.1 Da (calcd 5668.6 Da, average isotopes). (E) Crude product after second ligation with an observed mass of 9213.0 ± 0.1 Da (calcd 9213.9 Da, average isotopes). (F) Crude product **13** after the desulfurization with an observed mass of 9148.6 ± 0.2 Da (calcd 9149.9 Da, average isotopes). (G) Purified Ub-DHA probe **14** with an observed mass of 9115.0 ± 0.1 Da (calcd 9115.9 Da, average isotopes).

With both the probes in hand, we then examined selectivity for labeling USP2 compared to USP7—a member of the USP family with high active site similarity to USP2 (Figure 5). We therefore treated our synthetic probes **14** and **17** with the recombinant USP2 and USP7 DUBs and compared their labeling efficiency by fluorescent gel. The labeling experiments showed that the USP2 reacted with Ubv2.3-DHA (**17**) three times more efficiently than with Ub-DHA (**14**) (Figure 5B). On the other hand, USP7 almost exclusively labeled Ub-DHA (**14**) and showed negligible labeling of the Ubv2.3-DHA (**17**) (Figure 5B). We then proceeded to examine this selectivity in a more complex environment by performing the labeling experiment in cellular lysate. For this study, we chose U2OS cell lines, as they are known to express substantial levels of USP7 and have minimal expression of USP2.[37] Both synthetic probes **14** and **17** were incubated with the

cell lysates with or without addition of recombinant USP2 and both demonstrated the appearance of a new fluorescent band after addition of USP2 corresponding to the covalent DUB-Probe formation (Figure 5C). Ubv2.3-DHA, **17** demonstrated mostly USP2 labeling and increased selectivity when compared to the Ub-DHA, **14**. We also observed a side reaction corresponding to the labeling of endogenous USP7 with each probe as was confirmed by western blotting with anti-USP7 (Figure 5C). Our strategy can substantially simplify the synthesis of existing complex probes such as Ub-like (Ubl) e.g. SUMO and ISG.

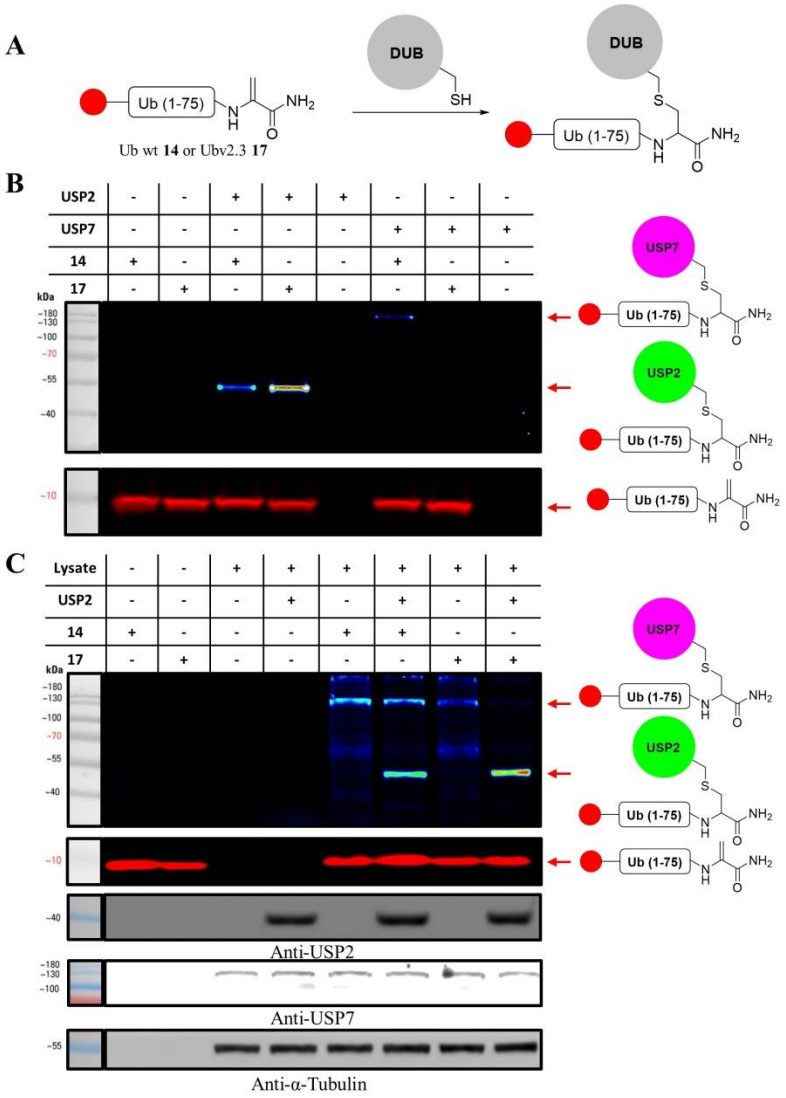


Figure 5. Fluorescent gel of the labeling experiment between two Ub-DHA probes and USP2 / USP7. (A) Schematic reaction between Ub-DHA probes and DUBs of the USP family. (B) Fluorescent gel of the reaction between Ub-DHA probes and purified USP2 and USP7. Top part is an intensity heat map based on TAMRA fluorescence demonstrating the formation of covalent DUB-Probe adducts. Bottom part is the unmodified probe from the same gel as a control for probe loading. (C) Fluorescent gel and western blot of the reaction between Ub-DHA probes and USP2 in U2OS cell lysate with and without USP2. Top part is an intensity heat map based on TAMRA fluorescence

demonstrating the formation of covalent DUB-Probe adducts followed by the fluorescent image of unmodified probes. Bottom is western blot against USP2, USP7 and α-Tubulin (loading control).

Temporal protection of native cysteines to enable selective desulfurization in protein semisynthesis

Our next goal was to employ maleimide in protein semisynthesis by using it as a temporal PG for a native Cys residue in a peptide thioester following one-pot NCL/desulfurization and final maleimide removal (Figure 6).[38] However, the protection of free Cys in the presence of a thioester could be challenging as it might lead to thioester hydrolysis. To test this, we synthesized two model peptides **19**, LYRAGC(*N*-Me.Mal)LYRAG-MMP (MMP=methyl 3-mercaptopropionate), bearing C-terminal thioester and **20**, Cys-LYRAGLYRAG, bearing *N*-terminal free Cys. Initially, we performed Cys protection in the thioester fragment, **18**, at different conditions and found that performing the reaction at -18 ^{o}C gave the desired maleimide protected thioester, **19**, without any detectable hydrolysis (Figure S17A). These two peptide segments were then ligated to give **21** and desulfurized to give **22** in the presence of imidazole[39] as a non-thiol catalyst and VA-044 for radical desulfurization respectively.[40] Similar to the SPCL results, the maleimide group was completely stable under imidazole NCL/desulfurization in solution. In the final step, the maleimide group on the Cys residue of **22** was removed quantitatively under our cleavage conditions to afford polypeptide **23** in 55% isolated yield.

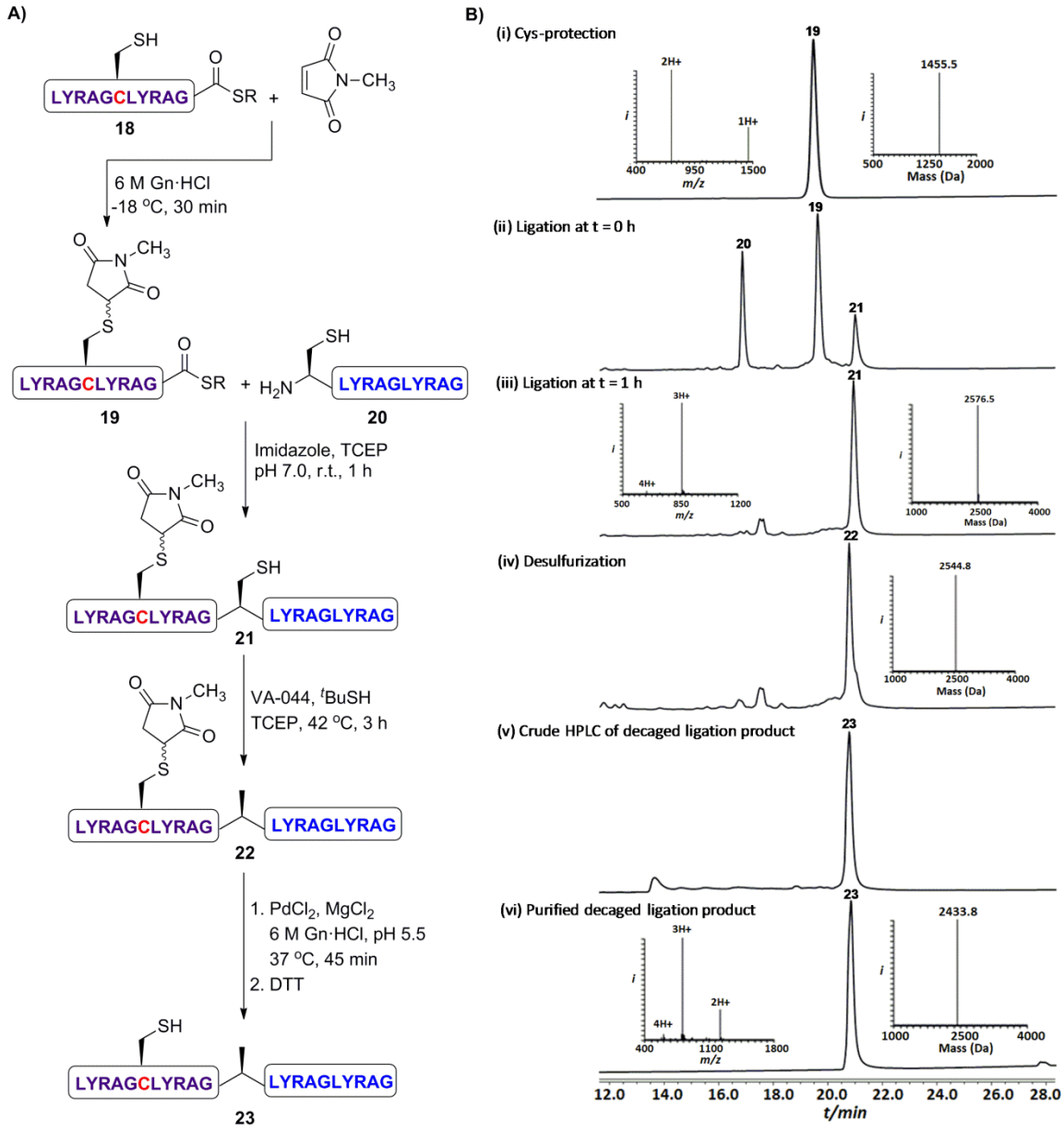


Figure 6. Examining the use of maleimide protection in solution and its stability during NCL/desulfurization. (A) The ligation strategy employing maleimide protection, one-pot ligation/desulfurization and cleavage the maleimide PG. HPLC-MS analysis: (B) One-pot NCL, desulfurization and maleimide removal. (i) Purified model peptide **19** with an observed mass of 1455.5 ± 0.1 Da (calcd 1455.5 Da, average isotopes). (ii) Ligation at t = 0 h, (iii) Ligation at t = 3 h with an observed mass of 2576.5 ± 0.1 Da (calcd 2576.9 Da,

average isotopes). (iv) Desulfurization product **22** with an observed mass of 2544.8 ± 0.1 Da (calcd 2544.9 Da, average isotopes). (v) Crude maleimide cleavage reaction. (vi) Pure polypeptide **23** with an observed mass of 2433.8 ± 0.1 Da (calcd 2433.9 Da, average isotopes).

Next, to apply these conditions for a more complex system such as peptides or proteins containing native Cys residues involved in intramolecular disulfide bonds and to check one-pot maleimide deprotection and disulfide bond formation, we used the oxytocin peptide as a model system (Figure S19). The maleimide protected oxytocin was treated under palladium cleavage conditions and subsequently quenched with DTC for 1 h.[16] The reaction afforded the desired disulfide bond formation in a one-pot reaction, highlighting the potential use of the maleimide PG for such an approach.

To apply our approach for production of a full semisynthetic protein, we chose *E.coli* Trx-1 enzyme (Figure 7). Trx-1, a thiol disulfide oxidoreductase which catalyzes the reduction of disulfide bonds in various proteins.[41] Our design for the semisynthesis of Trx-1 includes a recombinant fragment **24**, Trx-1 (2-93)-MESNA[42] (Figure S20) and a synthetic fragment **25**, Cys-Trx-1(95-109) (Figure S21). The recombinant peptide containing α-thioester can also be prepared by sortase A *via* an enzymatic approach.[43] The recombinant expressed fragment **24** bearing Cys33 and 36 was protected with the maleimide PG to form **26** and consecutively ligated[44] with fragment **25**. The ligation was completed after 4 h and product **27** was dialyzed against 6 M Gn·HCl to facilitate one-pot desulfurization.[38] A free radical desulfurization step and dialysis against 6 M Gn·HCl gave **28**. Finally, maleimide was removed from the desulfurized product **28** by Pd

treatment and subsequently quenched with DTC for 1 h to form the oxidized Trx-1, **29** in 34% isolated yield. Notably, the entire process of the 5 steps was achieved in one-pot. The semi synthetic Trx-1 exhibited the expected secondary structure[45] (Figure 7H) as shown by circular dichroism (CD) and the expected native enzymatic activity using the reported Trx-1 activity assay[46] (Figure 7J).

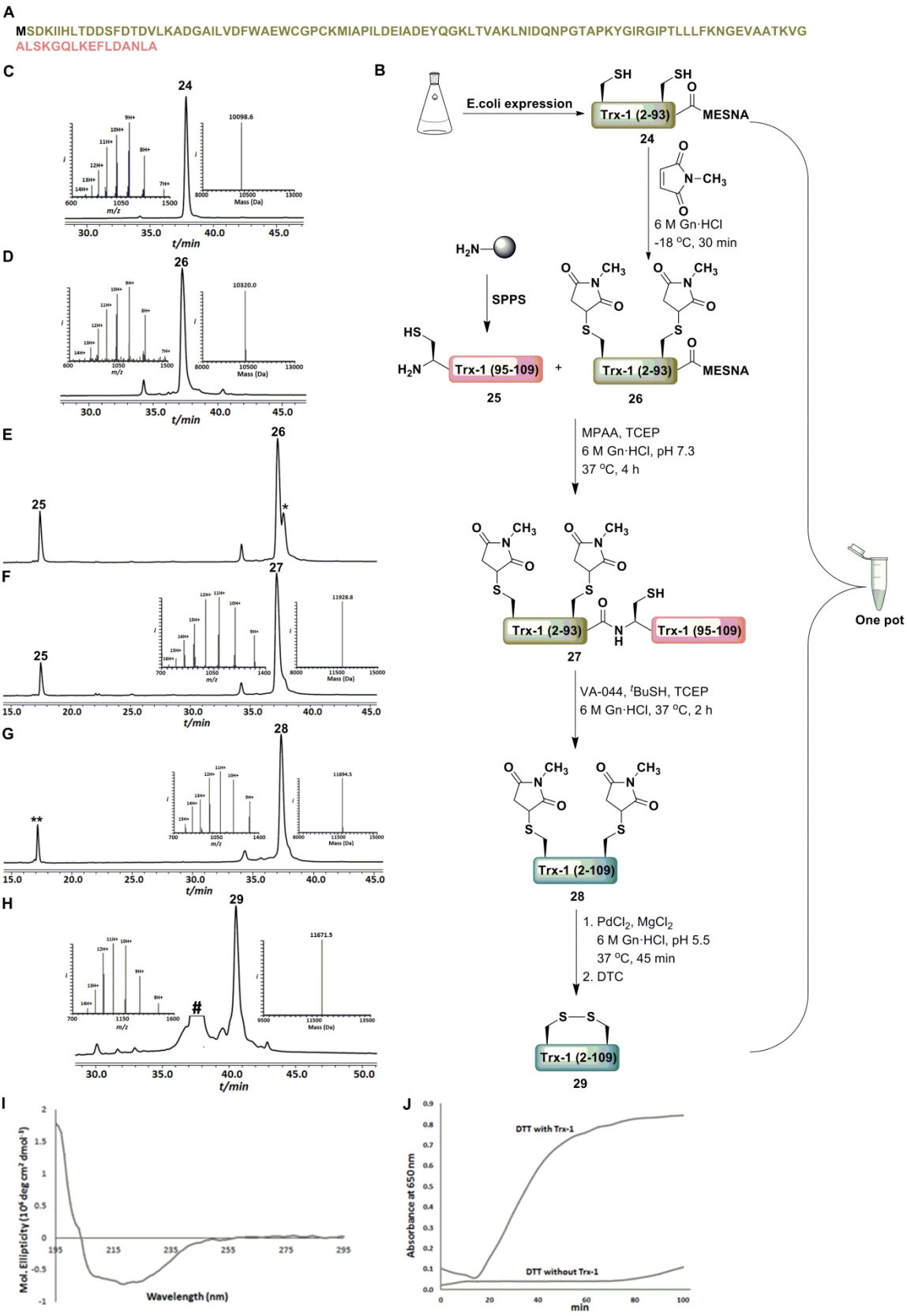

A
MSDKIIHLTDDSFDTDVLKADGAILVDFWAEWCGPCKMIAPILDEIADEYQGKLTVAKLNIDQNPGTAPKYGIRGIPTLLLFKNGEVAATKVG
ALSKGQLKEFLDANLA
B
E.coli expression
Trx-1 (2-93)
MESNA
24
6 M Gn·HCl
-18 °C, 30 min
SPPS
Trx-1 (95-109)
25
Trx-1 (2-93)
MESNA
26
MPAA, TCEP
6 M Gn·HCl, pH 7.3
37 °C, 4 h
Trx-1 (2-93)
Trx-1 (95-109)
27
VA-044, tBuSH, TCEP
6 M Gn·HCl, 37 °C, 2 h
Trx-1 (2-109)
28
1. PdCl2, MgCl2
6 M Gn·HCl, pH 5.5
37 °C, 45 min
2. DTC
Trx-1 (2-109)
29
One pot
C
24
10098.6
t/min
D
26
10320.0
t/min
E
25
26
*
F
25
27
t/min
G
**
28
t/min
H
29
#
t/min
I
Mol. Ellipticity (10⁴ deg cm² dmol⁻¹)
Wavelength (nm)
J
DTT with Trx-1
DTT without Trx-1
Absorbance at 650 nm
min

Figure 7. Examining maleimide protection for a recombinant fragment in aqueous solution and expressed protein ligation (EPL). (A) Trx-1 sequence (B) The ligation strategy employing one-pot maleimide protection, EPL/desulfurization, maleimide deprotection and disulfide bond formation. HPLC-MS analysis: (C) Trx-1 (2-93) recombinant fragment with an observed mass of 10098.6 ± 0.1 Da (calcd 10100.5 Da, average isotopes). (D) Maleimide protected Trx-1 (2-93) recombinant fragment with an observed mass 10320.5 ± 0.2 Da (calcd 10322.5 Da, average isotopes). (E) Ligation at t = 0 h. * is a MPAA-peptide related to thioester fragment. (F) Ligation at t = 4 h with an observed mass of 11928.8 ± 0.2 Da (calcd 11929.5 Da, average isotopes). (G) Crude desulfurization product with an observed mass of 11894.5 ± 0.8 Da (calcd 11897.5 Da, average isotopes). ** is an unidentified product related to the Cys fragment. (H) HPLC-MS analysis of the crude disulfide bond formation with the observed mass of 11671.5 ± 0.2 Da (calcd 11673.5 Da, average isotopes). # is a non-peptidic unidentified product related to DTC. (I) CD spectrum of semi-synthesized Trx-1. (J) Semi-synthesized Trx-1 activity assay for reduction of bovine insulin with and without addition of DTT. The change in turbidity of the reaction mixture was analyzed by plotting the absorbance at 650 nm versus time.

Conclusions

We have developed for the first time a practical method for the efficient removal of maleimide derivatives by using Pd^{II} complexes to enable peptide and protein (semi)synthesis. The synthetic utility of the present method was demonstrated by assembling Ub activity-based probes by SPCL, where the maleimide group was used as a cleavable linker. Pd^{II} treatment of the polymer support liberated the polypeptide

quantitatively with high yield and purity. In addition, we have shown that the maleimide group can be introduced as a protecting group in aqueous solution, especially for recombinant expressed fragments to enable EPL of complex semisynthetic targets. Further, our present protocol successfully facilitated one-pot maleimide detachment and disulfide bond formation under mild conditions. We envision the use of the maleimide group as an orthogonal protecting group and in cleavable solubilizing tags to facilitate peptide and protein synthesis. Furthermore, the maleimide group might find applications in chemical biology as a cleavable linker for anti-body drug conjugates and for proteomics studies.

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website.

Discussions of materials and methods used, peptide synthesis, removal conditions for maleimide group, orthogonal removal, solid phase chemical ligation, protection of cysteine for recombinant fragments, disulfide bond formation, HPLC and MS data, CD spectrum and enzymatic activity assay.

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Notes

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