Condensation of 2-((alkylthio)(aryl)methylene)malononitrile with 1,2-aminothiol as a novel bioorthogonal reaction for site-specific protein modification and peptide cyclization

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ABSTRACT: Site-specific modification of peptides and proteins has wide applications in probing and perturbing biological systems. Herein we report that 1,2-aminothiol can react rapidly, specifically and efficiently with 2-((alkyl**t**hio)(**a**ryl)**m**ethylene)**m**alononitrile (TAMM) under biocompatible conditions. This reaction undergoes a unique mechanism involving thiol-vinyl sulfide exchange, cyclization and elimination of dicyanomethanide to form 2-aryl-4,5-dihydrothiazole (ADT) as a stable product. An 1,2-aminothiol functionality can be introduced into a peptide or a protein as an *N*-terminal cysteine or an unnatural amino acid. The bioorthogonality of this reaction was demonstrated by site-specific labeling of not only synthetic peptides and a purified recombinant protein but also proteins on mammalian cells and phages. Unlike other reagents in bioorthogonal reactions, the chemical and physical properties of TAMM can be easily tuned. TAMM can also be applied to generate phage-based cyclic peptide libraries without reducing phage infectivity. Using this approach, we identified ADT-cyclic peptides with high affinity to different protein targets, providing valuable tools for biological studies and potential therapeutics. Furthermore, the mild reaction condition of TAMM condensation warrants its use with other bioorthogonal reactions to simultaneously achieve multiple site-specific modifications.

INTRODUCTION

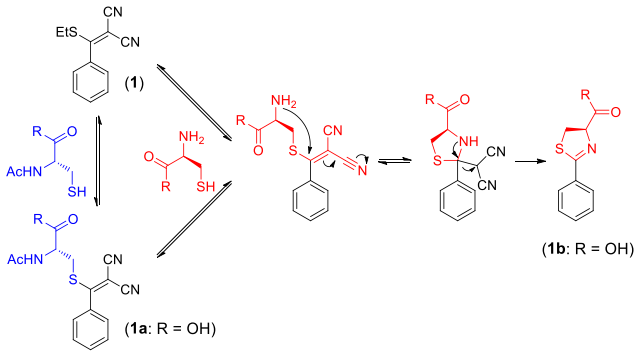
Site-specific modification is a powerful strategy for studying the structure and function of peptides and proteins.[1] To study polypeptides within their natural environment, various site-specific bioorthogonal reactions have been developed, including ketone condensations, alkyne-azide cycloadditions, Staudinger ligations, and Diels-Alder cycloadditions.[2] Despite the significant effort made, there are limitations associated with currently available technologies. Complex reagents that are expensive or difficult to prepare hamper potential applications; also, bulky labels often disrupt the polypeptide structure and hence function. Furthermore, the options for multiple modifications by using mutually orthogonal labeling systems are rather limited[3]. Importantly, many of the labeling systems are not compatible for live-cell labeling due to their slow reaction kinetics or lack of specificity or biocompatibility. Here, we present our effort of developing a specific, rapid and versatile labeling system involving a minimum functionality (*i.e.*, 1,2-aminothiol) easy to be introduced into chemically synthesized and recombinantly produced polypeptides and a reaction partner easily synthesized and tuned for its chemical and physical properties.

1,2-Aminothiol is a unique binucleophile that can be readily incorporated into a protein. Its small size warrants high atomic utilization efficiency and confers minimum perturbation to native proteins.[4] Consequently, this functionality has been used for site-specific protein labeling, semi-synthesis, and peptide cyclization.[2c, 5] Native chemical ligation relies on the 1,2-aminothiol group of a *N*-terminal Cys residue.[5e, 6] Though proven to be successful in protein synthesis, the reaction requires a labile thioester motif which is unstable in biological systems. 2-Cyanobenzothiazole can be used in 1,2-aminothiol labeling, and this reaction has been developed for protein labeling and functional macrocycle construction.[2c, 5a, 7] ~~Nevertheless, the condensation product (*i.e.*, luciferin) is not only bulky but hydrophobic, which can induce assembly or aggregation of short peptides.[7]~~ 1,2-Aminothiol can also react with benzaldehyde bearing an *ortho*-boronic acid substituent to promote formation of the corresponding thiazolidine derivative.[5c] However, aryl boronates are susceptible to rapid oxidation by hydrogen peroxide produced by living cells.[8] Novel biocompatible, robust and orthogonal 1,2-aminothiol labeling system with simple pre-requisite capable of modifying both small peptides and large proteins is still needed.

Here, we report the 2-((alkyl**t**hio)(**a**ryl)**m**ethylene)**m**alononitrile (TAMM)[9] condensation for 1,2-aminothiol labeling. In the TAMM reaction, a vinyl sulfide group is replaced by the thiol group of the 1,2-aminothiol (Scheme 1). Subsequently, Michael addition of the amino group takes place to form a thiazolidine, which induces the elimination of dicyanomethanide to

afford 2-aryl-4,5-dihydrothiazole (ADT). This reaction can be used for site-specific labeling *in vitro* as well as modification of proteins on the surface of live mammalian cells and phages without affecting their viability and infectivity. Furthermore, the TAMM condensation can be combined with phage display to identify high-affinity cyclic peptides against different targets. Together, these experiments highlight the versatility of the TAMM chemistry and its potentials in future chemical and synthetic biology research.

Scheme 1. Mechanism of TAMM condensation



RESULTS AND DISCUSSION

This work originated from our longstanding interest in developing dynamic vinyl sulfide bonds[10] using electron-deficient styrenes (*e.g.* TAMM) as the Michael acceptors.[11] While TAMM (*e.g.* **1**) undergoes thiol-vinyl sulfide exchange with thiol-containing molecules, such as acetylcysteine (Ac-Cys) to afford **1a** (Scheme 1), the reaction of **1** with Cys surprisingly formed a product with a mass of 66 Da lower than originally expected. Further ^{1}H and ^{13}C NMR characterizations revealed the product as 2-phenyl-4-carboxyl-4,5-dihydrothiazole (**1b**), suggesting irreversible elimination of dicyanomethanide and the formation of dihydrothiazole (Figure S1). Although dihydrothiazole derivatives can be synthesized through reactions of 1,2-aminothiols with aryl nitriles,[12] thionoesters or dithioesters,[13] these reactions have not been used for bioorthogonal labeling as they require either high temperature or labile reagents. On the other hand, in the TAMM condensation, both the reagent and the product are very stable (Figure S2), and the reaction can proceed either in the presence of a base (*e.g.* $NaHCO_3$) or in phosphate buffer (Figure S3). In addition, the exchangeability of the thiol leaving group in TAMM endows the reagent with a facile tunable nature in its chemical and physical properties. For example, a carboxyl functionality (*e.g.* **1a**) can be installed on the styrene scaffold to enhance the water solubility of TAMM. Specifically, **1a** can be isolated in high yield by reaction of **1** with Ac-Cys (Figure 1a). Similar to **1**, reaction of **1a** with Cys also led to formation of ADT product **1b**.

To investigate the bioorthogonality and reaction kinetics of the TAMM condensation, we examined the reaction of peptide **p1** in phosphate buffer (pH 7.4). Peptide **p1** was chosen as a model substrate as it contains not only an 1,2-aminothiol functionality at its *N*-terminal Cys but also a Lys residue of which the side-chain amino group can potentially cause side reactions. To our delight, reaction of **p1** with 2 equivalents of **1a** afforded only the desired product (**ADT-p1**) in quantitative yield (Figure 1b), indicating the bioorthogonality of this reaction. The second-order rate constant in phosphate buffer (pH 7.4) was calculated to be 4.2 M^{-1} s^{-1} (Figure S4), a value that is comparable to that reported for the reaction of 1,2-aminothiol with 2-cyanobenzothiazole.[2c] This was further confirmed by reacting **p1** with **1a** (2 eq) and 6-methoxy-2-cyanobenzothiazole (2 eq) at the same time, affording **ADT-p1** in about 60% yield (Figure S5). The bioorthogonality of TAMM condensation was further investigated using peptide **p2** containing both an *N*-terminal and internal Cys residues. Exclusive formation of the desired ADT product at the *N*-terminal Cys residue was observed when excess Ac-Cys was added into the reaction mixture to suppress the thiol-vinyl sulfide exchange of **1a** with the internal Cys residue (Figure 1c and S6). The reaction efficiency of **p2** with **1a** is also comparable to the reaction with 2-cyanobenzothiazole (Figure S5).

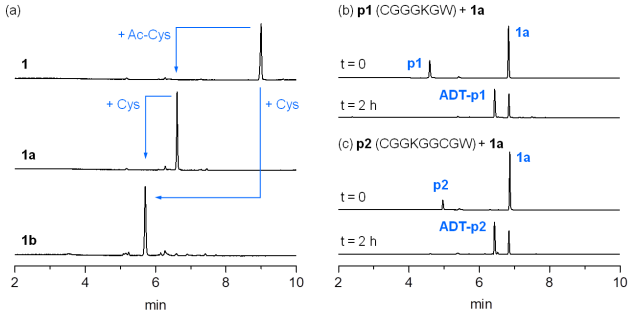


Figure 1. TAMM is a versatile motif for bioorthogonal reaction with 1,2-aminothiol. Chromatograms show absorbance at 280 nm. **(a)** ADT product **1b** (*m/z* for $[M+1]^+$ expected: 208.0432, found: 208.0427) can be formed by reaction of Cys with either **1** or **1a**. **(b, c)** Reaction of the designated peptide (50 μM) with **1a** (100 μM, 2 eq) in PBS (pH 7.4) for 2 h. **(b)** Peptide **p1** as the substrate to form **ADT-p1** (*m/z* for $[M+1]^+$ expected: 749.3188, found: 749.3218). **(c)** Peptide **p2** as the substrate in the presence of Ac-Cys (250 μM) to form **ADT-p2** (*m/z* for $[M+1]^+$ expected: 909.3494, found: 909.3605).

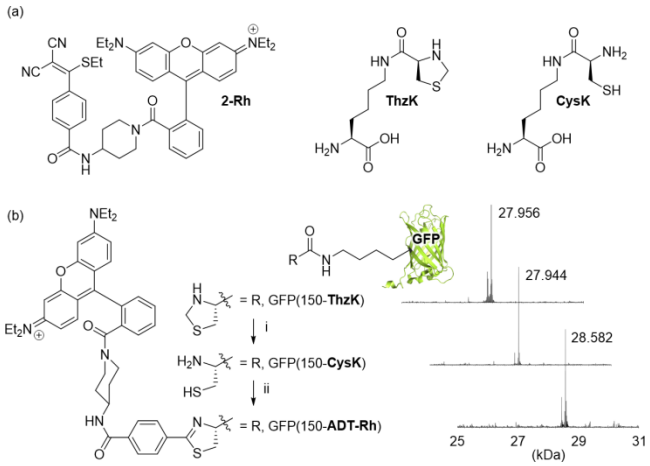


Figure 2. Site-specific labeling of a purified protein *in vitro*. **(a)** Structure of compounds. **(b)** Reaction scheme and mass spectra. Reagents and conditions: (i), 200 mM methoxyamine, 50 mM phosphate buffer (pH 6.0), 150 mM NaCl, 2 mM TCEP; (ii), 100

μM 2-Rh, 50 mM phosphate buffer (pH 7.4), 150 mM NaCl, 500 μM TCEP.

1,2-Aminothiol can also be added into a protein by genetic code expansion for TAMM labeling.[5a] To demonstrate this feasibility, His-tagged green fluorescent protein (GFP) containing a N^{ε}-L-thiaprolyl-L-lysine (**ThzK**) residue at the 150th amino acid residue was prepared (Figure 2). Treatment of GFP(150-**ThzK**) with methoxyamine converted the thiazolidine into 1,2-aminothiol to yield GFP(150-**CysK**), which can be modified with a TAMM-rhodamine conjugate (**2-Rh**). The success in fluorescent labeling was evident by both mass spectroscopy (Figure 2) and in-gel fluorescence analysis (Figure S7). In addition, GFP(150-**CysK**) can also be labeled with other TAMM-fluorophore conjugates (Figure S7).

Proteolytic process is another approach to generate 1,2-aminothiol functionality for TAMM labeling.[2c] We therefore explore this strategy to label cell-surface proteins on mammalian cells with TAMM-fluorophore conjugates. For example, TEV protease can recognize ENLYFQC sequence and cleave the amide bond between Gln and Cys, generating a *N*-terminal Cys residue. Accordingly, a plasmid expressing a fusion protein containing a *N*-terminal extracellular-targeting sequence, followed by the TEV recognition sequence ENLYFQC, fluorescent protein mCherry and a transmembrane domain, was constructed (Figure 3a). Live transfected HEK cells were incubated with the labeling solution containing both TEV protease and TAMM-fluorophore conjugate **2-BDP** (Figure 3b) for 30 minutes. Subsequent washing removed the unreacted conjugate before imaging. Live-cell fluorescence imaging in the mCherry channel confirmed the membrane expression of the fusion protein (Figure 3). In addition, fluorescence from **2-BDP** clearly superimposed with mCherry fluorescence (Pearson's correlation coefficients = 0.92), indicating site-specific fluorescent labeling. This was further confirmed by in-gel fluorescence (Figure S8). Using this approach, we were able to selectively modify cell-surface proteins with different TAMM-fluorophore conjugates (Figure S8).

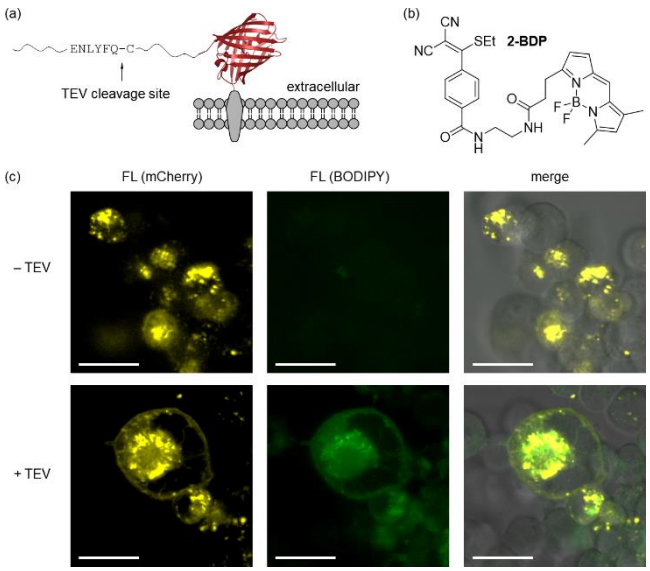


Figure 3. Site-specific labeling of a cell-surface protein on mammalian cells. **(a)** Generation of a *N*-terminal Cys for TAMM condensation by proteolysis. **(b)** Structure of **2-BDP**. **(c)** Confocal microscopy images showing TEV-dependent labeling. Scale bars represent 20 μm.

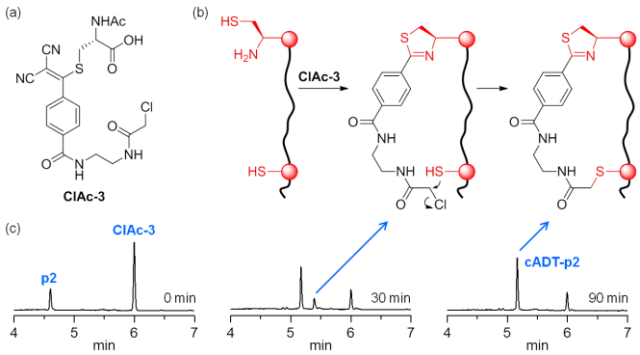


Figure 4. TAMM-mediated peptide cyclization. **(a)** Structure of **ClAc-3**. **(b)** Cyclization of a peptide bearing a *N*-terminal Cys and an internal Cys residues. **(c)** Reaction of **p2** (50 μM) and **ClAc-3** (100 μM) in phosphate buffer (pH 7.4) in the presence of Ac-Cys (200 μM) to form **cADT-p2** (*m/z* for $[M+2]^{2+}$ expected: 518.1998, found: 518.1125).

The TAMM reaction can be applied to labeling N-terminal cysteine, and this is further demonstrated by applying it in a coupled reaction that generates cyclic peptides. TAMM containing a chloroacetyl group (*e.g.* **ClAc-3**, Figure 4a) could be applied to cyclize peptides and proteins containing both an *N*-terminal and an internal Cys residues. This is because the chloroacetyl group is considered inert to intermolecular reactions with thiols.[14] Nevertheless, after the *N*-terminal TAMM condensation, it can undergo intramolecular reaction with the thiol group on the internal Cys residue (Figure 4b) as the proximity effect can accelerate reactions up to 10^5 folds.[15] To avoid the unwanted exchange reaction between internal Cys residue and the vinyl sulfide functionality on the TAMM reagent, as well as possible oxidation of Cys residues, 4 equivalents of Ac-Cys and TCEP were included in the reaction mixtures. Under this condition, model peptides, **p2** and **p3**, of different loop lengths were cyclized in high yields upon treatment with **ClAc-3** to afford the corresponding ADT-cyclic peptides (Figures 4b and S9). The cyclization primed by TAMM condensation has advantages in terms of biocompatibility and specificity compared to other crosslinking reagents with two highly reactive groups to free thiols, such as 1,3-bis(bromomethyl)benzene (BBMB).[3a, 16]

Encouraged by the success in cell-surface protein labeling and peptide cyclization, we decided to combine TAMM condensation and phage display to screen ADT-cyclic peptides binding to different protein targets. We designed a library of peptides displayed at the *N*-terminus of pIII protein on M13 phages.[17] After cleavage of the signal sequence by the endogenous enzyme in *E. coli*,[18] the peptide-pIII fusions will contain an *N*-terminal and an internal Cys residues separated by nine random amino acids encoded by NNK, forming the CX9C library, where X is any amino acid (Figure 5a). Display of peptides with nine randomized residues can generate quality

peptide libraries with sufficient content (>10^9) and reasonable coverage of sequence space.

We first used a biotin-containing TAMM (**Biotin-3**) to optimize reaction conditions for TAMM condensation. Under the optimized condition, phages were first treated with 1 mM TCEP for 30 min to reduce disulfide bonds, followed by addition of 1 mM **Biotin-3** and 2 mM Ac-Cys for 2-h incubation. Using a biotin-streptavidin pull-down assay,[19] this one-pot procedure reproducibly gave > 90% yield for TAMM condensation (Figure 5b). In contrast, there is no significant difference in pull-down efficiency of (i) phages without treatment of **Biotin-3** (*i.e.* blank control as signals were resulted from non-specific binding to streptavidin), (ii) phages without pretreatment with TCEP, (iii) phages treated with TCEP and **ClAc-3** before the addition of **Biotin-3**, and (iv) phages without an *N*-terminal Cys residue (*e.g.* GCXCX5CX5C, from *N*-to-*C* terminus; termed GC4 library).

Chemically cyclized peptide libraries are particularly useful for selecting lead peptides by phage display.[16c, 20] A conventional crosslinking reagent, BBMB, can be used, but it causes >2 orders of magnitude reduction in phage infectivity (Figure 5c), likely due to nonspecific crosslinking of native cysteine residues in phage protein pIII.[16c, 21] This consequently decreases the library size and hampers the chance of isolating a "hit". To our delight, cyclization using **ClAc-3** has no effect on phage infectivity (Figure 5c). This highlights the advantage of TAMM condensation in constructing cyclic peptide libraries.

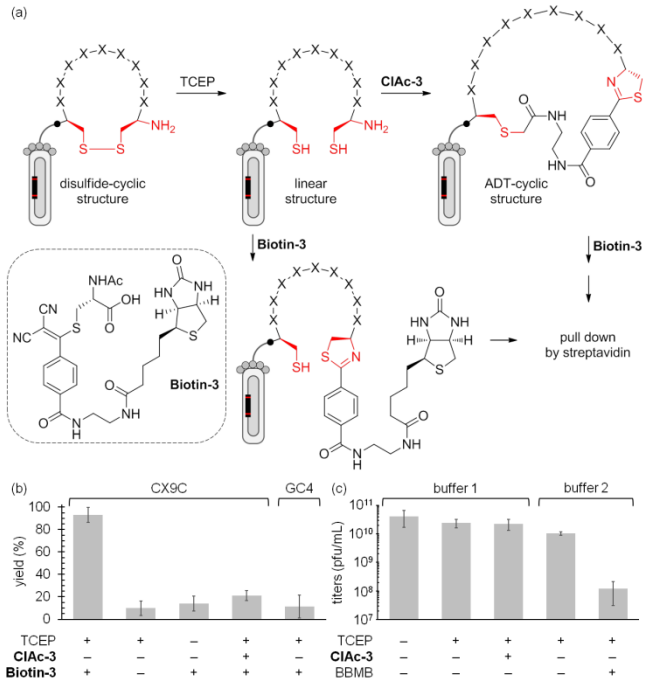


Figure 5. Cyclization of peptides displayed on the surface of phages using **ClAc-3**. **(a)** Scheme for the experimental operations. **(b)** Using streptavidin pull-down assay to characterize the TAMM condensation efficiency. Phages can only be pulled down efficiently with **Biotin-3** when they have a free N-terminal Cys residue. Control phages (GC4) have an N-terminal Gly residue. **(c)** Infectivity of phages under different conditions. buffer-1: 10 mM PBS (pH 7.4); buffer-2: 20 mM NH_4HCO_3 (pH 8.0), 5 mM EDTA.

The CX9C library was then used for screening high-affinity cyclic peptide ligands to different protein targets. Anti-apoptotic protein Bcl-2 was selected as the first target, which is closely associated with cancer.[22] A truncated Bcl-2/Bcl-xL chimera was expressed as a fusion protein with a Sumo-His-tag.[23] This protein was biotinylated and immobilized on magnetic beads with streptavidin, to which the ADT-cyclic phages were applied. After three rounds of panning, 30 phage clones were randomly picked and sequenced, giving 12 unique amino acid sequences with a consensus sequence of CPXRYXWDXXC (Figures 6a and S10). However, drastically different results were obtained from the disulfide-cyclic phages (no treatment of TCEP and **ClAc-3**, Figure 5a) and the linear phages (only treated with TCEP but not **ClAc-3**, Figure 5a) under the same conditions. Different consensus sequences found in the two controls highlights that the ADT-cyclization enables the display of unique cyclic conformations on phage surface that are not available in conventional linear and disulfide-cyclic peptide libraries. The binding of three phage clones (**CB-4**, **CB-5**, **CB-7**) to Bcl-2 was tested using ELISA, and TAMM-mediated cyclization was shown to be crucial for binding of phages to Bcl-2 (Figure 6b).

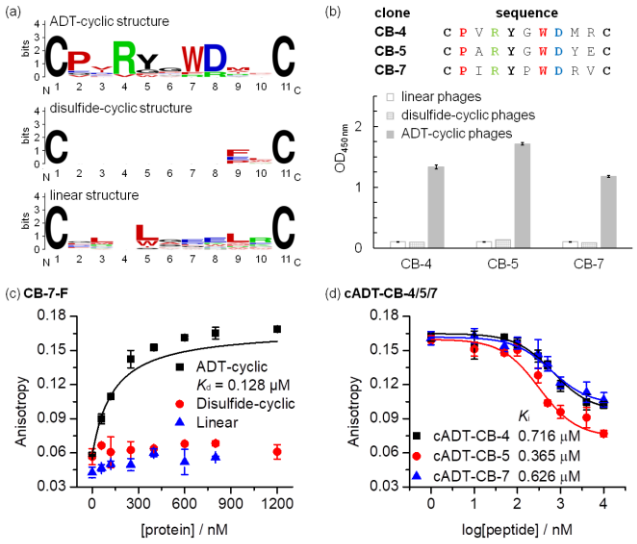


Figure 6. TAMM-mediated cyclization enables selection of ADT-cyclic peptides with high binding affinity. **(a)** WebLogo[24] showing consensus sequences obtained from the panning of phages with either ADT-cyclic, disulfide-cyclic or linear peptide structure against Bcl-2. **(b)** Binding of three phage clones (**CB-4**, **CB-5**, **CB-7**) to immobilized Bcl-2. Bound phages were detected with an HRP-conjugated anti-M13 antibody. **(c)** Binding of ADT-cyclic, disulfide cyclic or linear **CB-7-F** to Bcl-2 determined using a fluorescence polarization assay. **(d)** Binding of **cADT-CB-4/5/7** to Bcl-2 by competition with **cADT-CB-7-F** in a fluorescence polarization assay.

The three peptides were then chemically synthesized and cyclized using **ClAc-3**. To quantify the binding affinity, a lysine-conjugated fluorescein (**F**) was attached to the C-terminus of peptide **cADT-CB-7**. The dissociation constant (K_d) of the

resulting **cADT-CB-7-F** to Bcl-2 was determined to be 0.128 μM by fluorescence polarization. Neither the disulfide-cyclic **CB-7-F** nor its linear counterpart exhibits a measurable binding affinity (Figure 6c). The affinity of non-fluorescent **cADT-CB-4/5/7** to Bcl2 was measured in competition with **cADT-CB-7-F** by monitoring fluorescence polarization. All three peptides exhibited a submicromolar affinity to Bcl-2 (Figure 6d), whereas disulfide-cyclic and linear **CB-4/5/7** showed negligible affinity (Figure S10). In addition, two peptides that were most abundantly enriched from selections of linear and disulfide-cyclic libraries respectively were synthesized and labeled with fluorescein for affinity characterization and comparison. While the disulfide-cyclic peptide exhibits negligible binding to Bcl-2, the linear peptide can bind to Bcl-2 with a dissociation constant of 0.062 μM (Figure S11). ~~The binding site of the linear peptide on the surface of Bcl-2 is very likely different from that of the ADT-cyclized peptide as their sequences share no obvious consensus, though there is interplay between the two binding sites as demonstrated by competition fluorescence polarization (Figure S9).~~ The high affinity of the linear peptide suggests the possibility of selecting new cyclic peptide ligands capable of binding with Bcl-2 protein by varying the structure or length of ADT crosslinks. These results, taken together, also encourage further studies on both structures of peptide-protein complexes and screening of ADT-cyclic peptide libraries with new crosslink structures. Currently, development of peptide ligands and therapeutics to Bcl-2 family proteins has been an emerging frontier.[25] Available peptide binders to Bcl-2 are mainly stapled peptides of ≥20 residues derived from the Bcl-2 homology domains.[25] These peptides have an α-helical structure stabilized through hydrocarbon crosslinkers. While displaying no sequence homology to the previously reported stapled peptides,[25a] our ADT-cyclic peptides represent interesting alternatives for future studies due to their smaller size and ease of preparation.

The general applicability of the TAMM-mediated cyclization in phage display was further demonstrated by using two other proteins, Keap1 and Mdm2, as the immobilized targets. Both proteins are potential therapeutic targets for various diseases, including cancers, autoimmunity diseases, and inflammatory diseases.[26] After selections, consensus sequences were obtained for each target (Figure S12), and the identified ADT-cyclic peptides showed submicromolar binding affinities (Figures S13). For selection of cyclic peptide ligands towards many other targets of therapeutic interest, both the structure of ADT-crosslinker and the number of randomized amino acid residues can be fine-tuned for optimization.

CONCLUSION

In summary, we have described a novel condensation reaction for the labeling of 1,2-aminothiol functionality on peptides and proteins *in vitro* as well as on cell and phage surfaces. TAMM condensation represents a useful alternative to existing approaches for protein labeling. It is compatible with physiological conditions and proceeds with a high degree of specificity and efficiency. On the other hand, TAMM conjugates containing a chloroacetyl group can cyclize peptides with an *N*-terminal and an internal Cys residues. This strategy is applicable to phage display technique without reducing phage infectivity. Using this approach, we identified monocyclic peptides with high affinity to different protein targets, which are closely associated with different human diseases, providing valuable tools for biological studies and potential therapeutics. Moreover, it provides a new perspective of designing orthogonality to existing strategies for cyclic peptide construction[27] and can be combined with recent advance of genetic code expansion in phage display,[28] generating peptide libraries with more complex topologies.

ASSOCIATED CONTENT

Supporting Information

Experimental details for the chemical synthesis, cloning, biological experiments; ^{1}H and ^{13}C NMR spectra of all new compounds; in-gel fluorescence images of proteins; sequences of selected peptides. This material is available free of charge via the Internet at http://pubs.acs.org).

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Notes

The authors declare no competing financial interests.

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