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A sulfonium tethered peptide ligand rapidly and selectively modifies protein cysteine in vicinity

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Significant efforts were invested to develop site-specific protein modification methodologies in the past two decades. In most cases, a reactive moiety was installed onto ligands with the sole purpose to react with specific residues in proteins. Herein, we report a unique peptide macrocyclization method via the bis-alkylation between methionine and cysteine to generate cyclic peptides with significantly enhanced stability and cellular uptakes. Notably, when the cyclized peptide ligand selectively recognizes its protein target with a proximate cysteine, a rapid nucleophilic substitution could occur between the protein Cys and the sulfonium center on the peptide to form the conjugate. The conjugation reaction is rapid, facile and selective, triggered solely by proximity. The high target specificity is further proved in cell lysate and hints its further application in activity based protein profiling. This method enhances the peptide's biophysical properties and generates a selective ligand-directed reactive site for protein modification and fulfills multiple purposes by one modification. This proof-of-concept study reveals its potential for further broad biological applications.

Introduction

Site selective protein conjugation provides controllable tools to directly and precisely analyze protein functions in important cellular processes¹. Thus, multiple approaches have been developed to selectively modify endogenously reactive amino acid residues in proteins²⁻⁵. Chemo-selective reactions on particular residues in proteins are broadly utilized, including cysteine⁶⁻⁹, lysine¹⁰⁻¹⁵, tyrosine¹⁶, tryptophan¹⁷, arginine¹⁸ and methionine^{19,20}. The development of tools for site-selective protein labeling is in high demand due to its high precision and versatility. These tools include the genetic incorporation of unnatural amino acids within proteins equipped with "bioorthogonal" reactivity^{21,22}, genetic incorporation of peptide sequences(tag) for spatial recognition²³ and the utilization of N-terminal/C-terminal sites for protein labeling²⁴.

Another alternative approach for regio-selectivity is the ligand-induced protein conjugation which is mainly based on a precisely spatial positioning between a functional group of ligand and a reactive residue in protein. Meares et al. pioneered this concept in developing antibody conjugation with infinite affinity²⁵. Recently, Hamachi et al. established the ligand-directed (LD) chemistry to specifically label protein of interest (POI) in living cells^{26, 27}. Howarth et al. reported the Spytag-Spycatcher system in which the 13-

residue Spytag peptide was spontaneously and selectively form an isopeptide bond with the Spycatcher-tagged proteins²⁸. These ligand-directed approaches provide promising opportunity to balance the reactivity and selectivity for protein modification.

Regarding the selection of amino acid residues for ligand-induced protein conjugation, the high nucleophilicity and low abundance of cysteine in proteins makes it a prime residue for selective protein conjugation. In general, cysteine residues were let to i) react with electrophilic moieties such as haloalkyl or alkenyl groups; ii) undergo metal assisted reactions and iii) be converted into dehydroalanine for further modifications. The diverse methods for selective protein labeling have been widely used in the study of post-translational modifications(PTMs), cellular imagine, activity based protein profiling (ABPP) or covalent drug discover^{2, 29-31}. Notably, covalent inhibitors of key kinases were recently approved by FDA as efficient cancer therapeutics, such as ibrutinib and rociletinib³²⁻³⁴.

Peptides are recently utilized as promising ligands for covalent protein modification due to their high binding affinity, selectivity and biocompatibility. For example, Xia et al. installed a mildly electrophilic α -chloroacetyl moiety onto peptide ligands for covalent conjugation with cysteine near protein-peptide interaction sites³⁵. Walensky and Fairlie et al. independently developed covalent BFL-1 inhibiting stapled peptides with an additional electrophilic warhead acrylamide³⁶⁻³⁸. Wang et al incorporated the aryl sulfonyl fluoride (Ar-SO₂F) to the SAHp53-8 peptide that interrupted p53-Mdm2/4 interactions³⁹. To the best of our knowledge, all reported methods require a pre-arranged reactive moiety solely for the purpose of conjugation. However, the reactive group either requires special steps to prepare or may undergo non-specific reactions. For peptides such as the BFL-1 ligands, additional stapling step is also necessary for enhanced stability and cellular uptake. Thus, we are seeking to develop a facile peptide cyclization method which could enhance the peptides' stability and cellular uptakes, meanwhile

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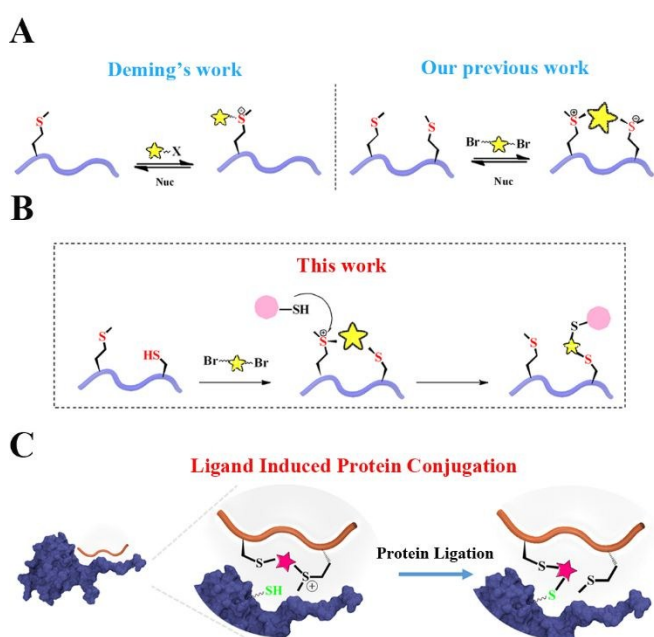
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the modification also generates a highly selective reaction site for reactive amino acid residues in protein.

Based on Deming's pioneering work of selective methionine alkylation, we recently developed a bisalkylation modification of Met to generate cyclic peptides with better cellular uptake and stability^{40, 41}. Taking the current protein labelling demands into consideration, we envisioned this method could be further developed into a novel and facile methodology to fulfil multiple purposes mentioned above. As shown in Scheme 1, the bis-alkylation between Cys and Met could generate a cyclic peptide with a tuneable tether and an on-tether sulfonium center, which could help on peptide' stability and cellular uptakes. The labile sulfonium center may further undergo proximity promoted Cys substitution with available Cys in the vicinity of the POI's binding pocket. Based on previous reports and our own findings, the sulfonium centers are stable in the presence of thiols for a reasonable time, indicating their potential cellular application^{20, 42}.



Scheme 1. Schematic presentation of Cys-Met bis-alkylation and proximity-promoted protein labeling with sulfonium tethered peptide. (A) Deming et al.'s reports of selective methionine alkylation and our previous report of chemoselective methionine bis-alkylation. (B) Peptide macrocyclization through cysteine and methionine bis-alkylation. (C) Ligand induced protein conjugation using the Cys-Met macrocyclization methods.

Results and discussion

To meet the demands for both peptide stapling and protein modification, we first used a model hexapeptide (1) to assess the efficiency of peptide cyclization shown in Figure 1A. The peptides were constructed via conventional Fmoc-based solid phase peptide synthesis. The cyclization involved two steps: 1) the deprotection of Trt-protected cysteine and alkylation with di-halogenated linkers on resin; 2) the cleavage of the peptide from the resin to give the resulting bis-alkylated cyclic peptides. The alkylation of Met happened spontaneously during resin cleavage to generate the cyclic peptide (1-I) with a 86% conversion based on HPLC integration. The cyclization

product was further supported by LC-MS and ¹H-NMR with a clear shift of phenyl proton shown in Figure S1. We further confirm that the cyclization process happened during resin cleavage step instead of the DIPEA/DMF step shown in Figure S2. Notably, as the Met alkylation will generate a new on-tether chiral center, we successfully isolated two epimers as indicated in Figure S3. Then we tested the reaction efficiency of different di-alkylating linkers and found the peptide (1) reacted smoothly with different linkers to provide products with high conversions indicated in Figure 1A and Figure S3. The epimer ratios of different linkers were generally ~1:1 and in some cases, the peptide epimers were not separable under our HPLC conditions (Figure S3.). Notably, the sulfonium chiral center of the purified epimer is not very stable and would slowly racemize into the initial epimer mixtures. In addition, the separable epimers showed similar secondary structures with CD spectroscopy measurement, suggesting the chiral center had limited effect on peptide secondary structure (Figure S4). To assess the functional group tolerance of our method, ten peptides with different sequences were prepared as summarized in Figure 1B. All peptides efficiently generated the corresponding cyclic peptides with high conversion rates.

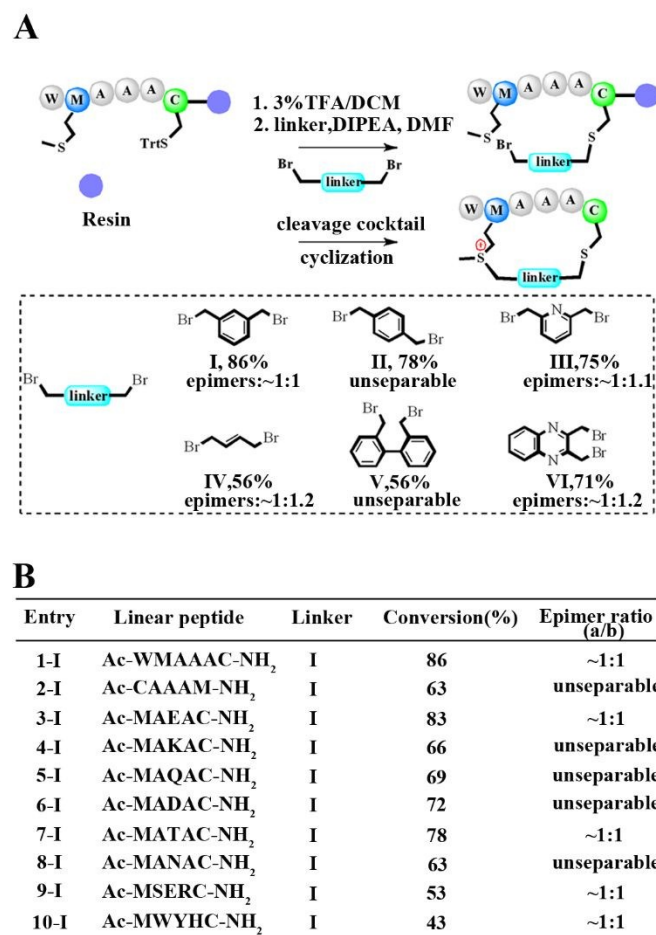


Figure 1. Facile construction of stabilized peptides by bisalkylation between Cys and Met. (A) Constrained peptide preparation and different linkers tested in this study. The Trt-protected cysteine was deprotected under 3%TFA in DCM until the yellow liquid no longer appeared. Then di-halogenated linker(2.0 equiv.) was added with DIPEA(4.0 equiv.) in DMF and was let react for 3 hours. Methionine alkylation and peptide cyclization was completed when the resin was cleaved under TFA cocktails(TFA: TIS: H₂O=95:2.5:2.5). Epimer ratio was calculated according to the HPLC traces. (B) Ten different peptides were tested for functional residue tolerance.



The Met alkylation was reported to be reversible in the presence of proper reductants under mild conditions (pH 7.4 PBS, 37°C)⁴⁰. Among the reductants and nucleophiles tested in previous studies, GSH was reported to be the least reactive⁴⁰. We first tested the reaction rate of sulfonium tethered peptides 1-1a (1mM) with 2-mercaptopyridine (10mM) in PBS buffer (pH 7.4) as shown in Figure 2A. The LC-MS analysis clearly showed the time-dependent reduction of peptide 1-1a (1mM) with 2-mercaptopyridine (10mM) in PBS (pH 7.4). We then tested the reaction rate of sulfonium tethered peptide 1-1a and 1-1b (1mM) in the presence of different reductants (10mM, 2-mercaptopyridine, thiourea or GSH) in PBS buffer (pH=7.4) shown in Figure 2B and Figure S5. The reduction experiments were tracked by LC-MS at different time points and GSH was confirmed to be the weakest reductant among the tested reagents. The dealkylation efficiency of different linkers and their epimers were further tested and summarized in Figure S6. The peptide epimers showed briefly similar kinetics. Notably, peptide 1 equipped with linker V showed the quickest dealkylation rate (Figure S6). Sulfonium center on Met is stable in 20mM thiourea as reported by Gaunt *et al.* The on-tether sulfonium centers shown in Figure 2B showed similar stability.²⁰ Serum stability of peptide 1 analogues were tested and the cyclic ones were found to have significantly enhanced serum stability (Figure S7). To test whether our tethering strategy could improve cellular uptakes, peptide 11 with three arginine residues was prepared and reactions were performed with different linkers I-VI. The reactions went smoothly with high conversions and the epimers were separated if possible. The A2780 cells (Figure 2C) and 293T cells (Figure S8) were treated with 10 μ M FAM-labeled peptides for 4 hours and then incubated with 0.05% trypan blue before FACS analysis⁴³. In both A2780 and 293T cell lines, the constrained peptides showed significantly increased cellular uptakes than linear peptide 11. Confocal microscopy images of A2780 cells indicated the different cellular distribution of peptides with different linkers (Figure S9). To sum up, the model peptides constructed with this method showed enhanced stability and cellular uptakes and could conjugate with physiologically relevant nucleophiles. Notably, the sulfonium center showed limited conjugation with GSH after 48 hours at physiologically-relevant concentrations.

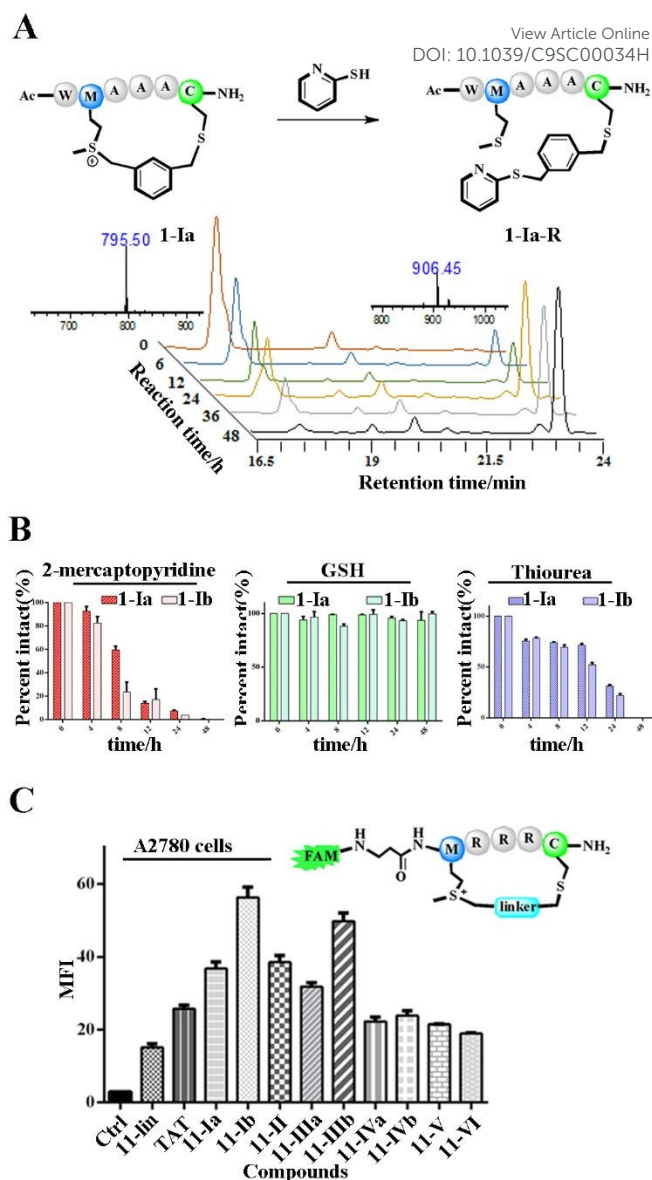


Figure 2. The biochemical properties of the constrained peptides. (A) Dealkylation of model peptide (1-1a) (1mM) in the presence of PyS (10mM) in PBS (pH=7.4) at 37°C for 48 hours. HPLC traces of the time dependent conversion between peptide (1-1a) and its conjugated product (1-1a-R). (B) The kinetics of peptide dealkylation reactions equipped with different reductants under the same reaction conditions as indicated in Fig 2A. (C) Cellular uptakes of the cyclic and linear peptides in A2780 cells treated with 10 μ M FAM-labeled peptides for 4 hours. All cells were incubated with 0.05% trypan blue for 3 minutes before further analysis.

To further prove the potential of this methodology for proximity induced cysteine modification, we constructed peptide ligands for PDZ^{ARG53} as shown in Figure 3A. PDZ^{ARG53} (PDZ^{ARG53} denotes the PDZ domain alone of PDZ-RGS3, and the protein sequence was shown in Figure S10) plays an important role in ephrin-B reverse signaling which is associated with SDF-1 (stromal derived factor 1) neuronal chemotaxis⁴⁴. PDZ^{ARG53} was reported to be covalently labeled at a cysteine by their peptide ligands bearing a chloroacetamide moiety and we envisioned it as an ideal showcase for this proof-of-concept study³⁵. There are three Cys residues (C33, C34, C73) in the vicinity of peptide ligand binding site of PDZ^{ARG53}³⁵. To study the peptide selectivity and site



selective cysteine conjugation of targets, a series of peptide ligands with different cyclization sites and different PDZ^{ARG53} mutants were then prepared including PDZ^{ARG53} C335C345 and PDZ^{ARG53} C735 (Figure 3A and Figure S10). We first tested the peptides' binding affinity of PDZ^{ARG53} and their mutants by fluorescence polarization assays shown in Figure 3A and Figure S11. The peptides with different stapling sites showed quite different binding affinity, while the Cys-Ser mutations (PDZ^{ARG53} C335/C345, PDZ^{ARG53} C735) appeared to have no obvious detrimental effects on peptide binding.

Then peptides PD1-I to PD3-I were reacted with PDZ^{ARG53} in pH 7.4 PBS (protein/peptide 20/100 μ M, 37°C, 1 hours). As shown in Figure 3B, peptide PD1-I with binding affinity of 16.87 μ M showed weak conjugation, while peptides PD2-I and PD3-I with Kd of 3.02 μ M and 1.1 μ M showed rapid covalent conjugation. Rationally, the scrambled peptide NS with no binding affinity for PDZ^{ARG53} couldn't conjugate with PDZ^{ARG53}, which further confirm the concept of ligand-induced protein conjugation. The protein conjugates still retained their secondary structure confirmed by Circular dichroism(CD) and thermal shift assays shown as Figure S12. We also found the reaction efficiency was reduced with the excess of competitive linear peptide confirming the ligand-induced conjugation (Figure S13). We then tested site selectivity of peptide PD3-I with wild type PDZ^{ARG53} and its mutants. Peptide PD3-I showed the most efficient reaction with wild PDZ^{ARG53}, and a little weaker reaction with PDZ^{ARG53} C735 but negligible reaction with PDZ^{ARG53} C335C345 (Figure 3B). The MS data further confirmed the satisfying covalent conjugation of PDZ^{ARG53} C735 and poor covalent conjugation of mutants PDZ^{ARG53} C335C345 (Figure S14-17), indicating the cysteine in the vicinity of protein-peptide interaction site was essential for ligand conjugation. The reaction kinetics and stoichiometric study between PDZ^{ARG53} and peptide PD3-I was then performed as shown in Figure 3C. The reaction started within 10 minutes and went to a ~80% conjugation within 60 minutes. One equiv. peptide was briefly enough to complete the conjugation. The kinetics and stoichiometric study clearly showed the efficient conjugation. We also found the reaction efficiency was reduced in acidic buffer but went more smoothly in the basic conditions (Figure S18). The resulted protein-peptide conjugate was stable in 10mM GSH condition for at least 12 hours in 37 °C with no leaks detected (Figure S18), which hinted its potential biological applications in antibody-drug conjugate or protein post-translational modifications.

Then protein selectivity was carefully examined by using other proteins containing free Cys residues like BCL2, MgrA and SrtA (Table S3). No conjugation was observed as shown in Figure 3D which indicating the site-selective cysteine modification of our designed peptides. To assess the ability of peptide PD3-I to label PDZ^{ARG53} in a complex proteome environment, 293T cell lysates(300 μ g) were spiked with PDZ^{ARG53}(10 μ g), and then treated with 50 μ M FAM labeled peptide PD3-I shown in Figure 3E as referring to the work of Sun et al.⁴⁵ The gel data showed a clear single fluorescent band with the right molecular weight indicating a clean and selective conjugation of peptide PD3-I to PDZ^{ARG53}. The successful conjugation was further confirmed by pull down assay using Ni-NTA Agarose Beads shown in Figure S19.

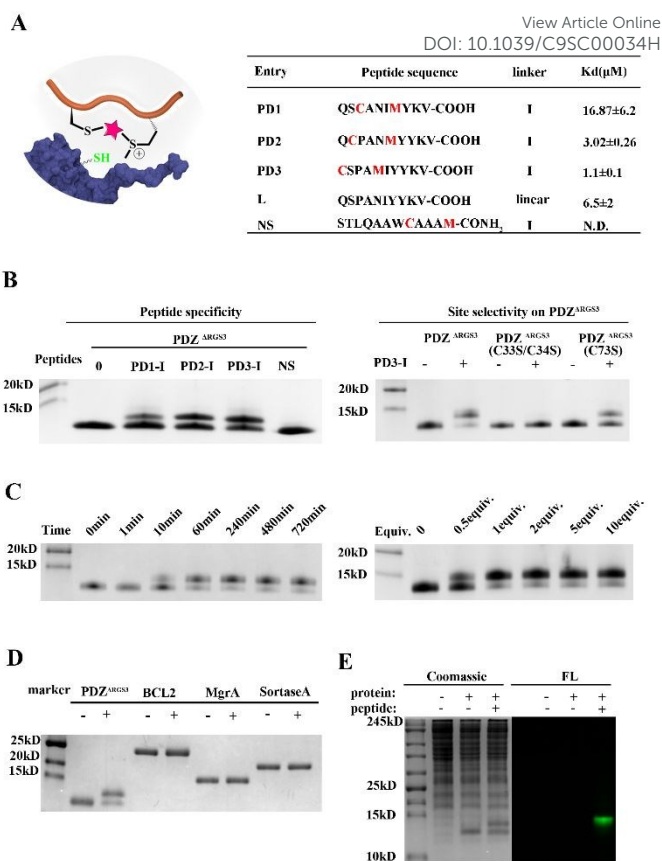


Figure 3 Design and synthesis of reactive peptide ligands for covalent PDZ^{ARG53} conjugation (PDZ^{ARG53} denotes the PDZ domain of PDZ-RGS3). (A) Peptide sequences designed for protein conjugation. (B) Peptide specificity and site selective cysteine conjugation of PDZ^{ARG53}. PDZ^{ARG53} was reacted with different peptides (protein/peptide 20/100 μ M, pH 7.4, 1 hour). PDZ mutants were incubated with peptide PD3-I (protein/peptide 20/100 μ M, pH 7.4, 12 hours). (C) Reaction kinetic study of peptide PD3-I (protein/peptide 15/75 μ M, pH 7.4) for 0min, 1min, 10min, 60min, 240min, 480min and 720min, respectively. Stoichiometric study of peptide PD3-I from 0.5 equiv. to 10.0 equiv. incubating with PDZ^{ARG53} for 4 hours. (D) Ligand induced protein conjugation. Other proteins containing free Cys would not react with peptide PD3-I (protein/peptide 15/75 μ M, pH 7.4, 12 hours). (E) FAM-labeled peptides (PD3-I, 50 μ M) and PDZ^{ARG53} (10 μ g) were incubated with 293T cell lysates (300 μ g) for 24 hours. FL, in-gel fluorescence scanning.

The peptide was then tested for conjugation efficiency in HA-PDZ^{ARG53} transfected cell lysates shown in Figure 4. Anti-HA western analyses revealed that peptide PD3-I could conjugate with PDZ^{ARG53} in concentration dependent manner while the scrambled peptide NS couldn't label this target even under 100 μ M for 8 hours incubation, as shown in Figure 4A. The reaction in cell lysates could started in 30min and reached high conjugation within 8 hours at room temperature with 50 μ M PD3-I, as shown in Figure 4B. The reaction efficiency can be competed by the linear peptide ligand L, indicating the reaction in cell lysates was conducted by the ligand-induced protein conjugation (Figure 4C). The successful conjugation in cell lysates showed its potential for future cellular application, such as cell imaging, the study of protein-protein interaction, the discovery of covalent inhibitors and activity based protein profiling.



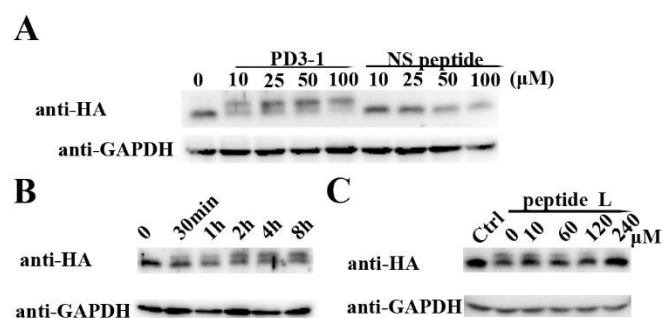


Figure 4. The covalent reaction in HA- PDZ^{ARG53} transfected cell lysates. (A) Covalent bonding of peptide PD3-1 or peptide NS to HA-PDZ^{ARG53} after 8h incubation at room temperature with HA-PDZ^{ARG53} transfected cell lysates. (B) Reaction kinetic study of peptide PD3-I and HA-PDZ^{ARG53} conjugation in HA-PDZ^{ARG53} transfected cell lysates for 0min, 30min, 1h, 2h, 4h and 8h respectively. (C) The high concentration of linear peptide competitively blocked the labeling of peptide PD3-1(30μM) to HA-PDZ^{ARG53} in cell lysates in dose-dependent manner.

As different linkers demonstrated different reaction rates (Figure S6), we then tested the conjugation efficiency of peptide PD3 equipped with different linkers as shown in Figure 5A. The linker IV showed the slowest cysteine conjugation rate. The successful conjugation was further confirmed by ESI-MS shown in Figure 5B and Figure S17, 20-22 which suggested that only one cysteine residue in PDZ^{ARG53} could be conjugated with the peptide. To further identify the modification sites, trypsin digestion of single reaction band cut from gel was sent for MS/MS analysis as shown in Figure 5C and Figure S23-25. The MS/MS results suggested an expected peptide fragmentation both containing peptide PD3-I and peptide segment containing Cys33, Cys34 or Cys73 of PDZ^{ARG53} indicating our peptide can selectively label PDZ in the vicinity of ligand binding sites.

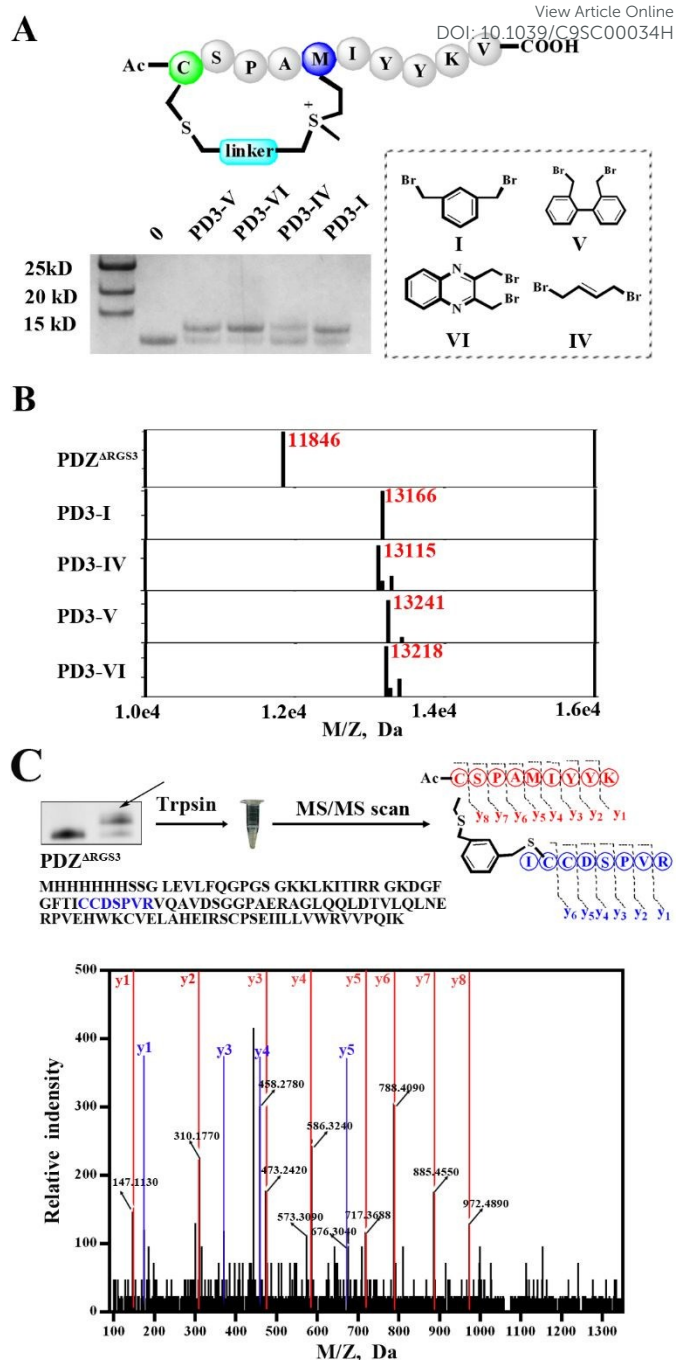


Figure 5. ESI MS analysis of the PDZ-peptide covalent conjugates. (A) The proteins were incubated with peptides equipped with different linkers (protein/peptide 15/75μM, pH 7.4, 12 hours). (B) The mass analysis of peptide-PDZ^{ARG53} conjugation, figures prepared by Prism based on original MS data shown in Figure S14-18. (C) Mass/mass spectrometry analysis of trypsin-digested PDZ-peptide conjugates indicating peptide PD3-I binds covalently to C33 or C34 in PDZ^{ARG53}.

Conclusions

In summary, we designed a facile macrocyclization method via bis-alkylation between cysteine and methionine with improved serum stability and cellular



uptake. Additionally, the tethered sulfonium could be a novel warhead for site selective protein modification in biocompatible conditions. To the best of our knowledge, it is the first attempt to combine peptide stapling and the installation of a selectively reacting moiety in one simple design. To demonstrate the principle, we constructed peptide ligands for PDZ^{ARG53} and clearly showed the reaction is rapid and efficient, highly selective and solely proximity-prompted. The reaction is viable in PBS, doesn't require any further special conditions and is selective in cell lysates. This method could have great potential in affinity-based protein profiling, target identification, and novel PPI modulator development.

Conflicts of interest

There are no conflicts of interest to declare

Licence to publish

Z. L, F. Y and D. W. prepared the manuscript and designed the research presented. D. W., M. Y. and Z. H. designed and performed the synthetic and biological experiments. N. L and C. L. prepared various proteins. R. Z. and W. L. help provide HPLC, MS and CD data. R.W and S.L. helped analyze the MS data. Y. J. and X. S helped prepare the cellular experiments.

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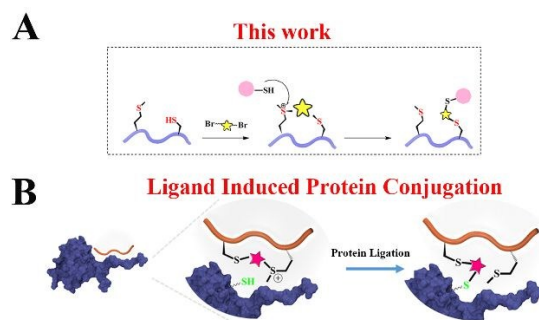


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This unique peptide stabilization method provide a tethered sulfonium that can rapidly and selectively modify protein cysteine in vicinity.

