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Thiazolidin-5-imine Formation as a Catalyst-Free Bioorthogonal Reaction for Protein and Live Cell Labeling

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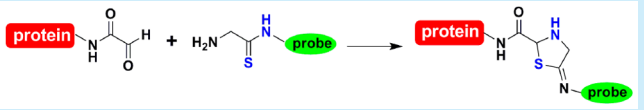
Supporting Information

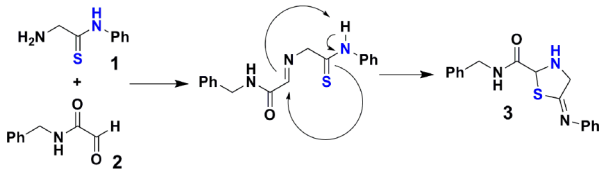
ABSTRACT: A previously undescribed reaction involving the formation of a thiazolidin-5-imine linkage was developed for bioconjugation. Being highly specific and operating in aqueous media, this simple condensation reaction is used to chemoselectively label peptides, proteins, and living cells under physiological conditions without the need to use toxic catalysts or reducing reagents.



Chemical modification of biomolecules such as proteins is extensively used to improve their physicochemical and pharmacological properties and to facilitate structure−function studies. Direct modification of proteins can be achieved by targeting the naturally existing functional groups of the amino acid residues such as the commonly used amino or thiol groups.[1] Although this strategy represents a straightforward way to modify proteins, it lacks site specificity due to the presence of multiple lysine and cysteine residues in a protein. To overcome this problem, uniquely reactive unnatural functionalities that are not present in the canonical amino acids, such as aldehyde, ketone, tetrazine, alkene, alkyne, and azide, are synthetically, biosynthetically, or enzymatically introduced into proteins. This makes it possible to precisely modify the protein via a chemoselective reaction between the unnatural functionality and a mutually reactive functional group on a modifying reagent.[2] To date, a variety of such mutually reactive pairs of functional groups have been developed for site-specific bioconjugation including the condensation between an aldehyde/ketone and an alkoxy-amine/hydrazine, the Staudinger ligation, the Cu (I)-catalyzed and strain promoted cycloaddition between alkynes and azides, the inverse electron demand Diels−Alder reactions between tetrazines and strained alkenes.[3] However, many of the available chemistries have their shortcomings such as synthetic difficulty to obtain the chemical probes containing the required orthogonal functionality, acidic reaction conditions that are incompatible for sensitive proteins, or the need for toxic catalysts or reducing reagents. Therefore, there is extensive interest in developing new biorthogonal and catalyst-free conjugation reactions that can be performed in aqueous solution under physiological pH. Herein, we describe a new condensation reaction that fulfills such requirements. We find that a 2-aminoethanethioamide moiety can react with an aldehyde group in a highly selective manner to form a thiazolidin-5-imine linkage. We show that this simple, yet previously undescribed reaction system is a useful bioconjugation method for the modification and labeling of peptides, proteins, and live cells.

We first demonstrated this reaction scheme in a model study using small-molecule compounds **1** and **2** (Scheme 1). Because

Scheme 1. Model Reaction between 1 and α-Oxo aldehyde 2 To Yield Condensation Product 3 and the Proposed Mechanism



of its poor solubility in water, **1** was allowed to react with **2** in PBS/acetonitrile/*t*BuOH mixture (2:2:5) at 37 °C. After 4.5 h, the product **3** was formed in ∼85% yield. Then **3** was isolated and characterized by NMR (^{1}H and ^{13}C) and mass spectrometry (Figures S1 and S2 in the Supporting Information (SI)). ^{1}H NMR analysis clearly assigned the peaks to the protons on C2 and C4 of the five-membered ring thiazolidin-5-imine structure of **3** (Figure S1). As a plausible reaction mechanism, the amine of **1** first reacts with the aldehyde to form the imine Schiff base, which was attacked by the sulfur of the thioamide tautomer to form the thiazolidin-5-imine linkage (Scheme 1). Clearly, the driving force here is ring formation, a mechanism that is similar

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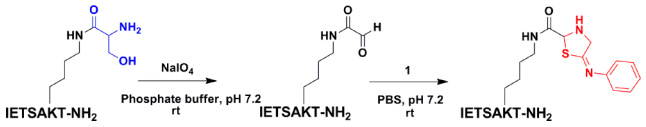
to the condensation reaction between an aldehyde and a 1,2-aminothiol moiety.[4] To test the stability of **3** in the aqueous media, we incubated **3** in the aqueous buffer at pH 4.5, 7.2, and 8.5 at 37 °C. At different time points (0, 2, 4, 8, 24, 72, 168 h), an equal aliquot of the sample was taken for HPLC analysis. Results show that **3** was quite stable in a neutral and slightly basic aqueous buffer (pH 7.2 and 8.5) in which only a small portion of **3** was hydrolyzed after 168 h at 37 °C (Figure S3 in SI). However, it was susceptible to hydrolysis in the acidic buffer at pH 4.5 as a significant degree of hydrolysis was observable after incubation for 2 h (Figure S3).

Encouraged by these results, we proceeded to test this reaction for chemoselective conjugation using a peptide. Therefore, we synthesized an aldehyde-containing peptide, H-IETSAK(X)T-NH_2, where X = α-oxo aldehyde, which was introduced via $NaIO_4$ oxidation of the 1,2-aminoethanol moiety of a Ser residue (Scheme 2 and SI). The condensation reaction

Scheme 2. Generation of α-Oxo aldehyde in Model Peptide and Its Subsequent Bioconjugation with 1



was conducted at intentionally high dilutions of the reactants (Scheme 2). Thus, the α-oxo aldehyde-containing peptide (0.1 mM) was incubated with **1** (0.2 mM) in PBS (pH 7.0) and the reaction was monitored by analytical HPLC and mass spectrometry. To our delight, after 1 h, the conjugation product could be observed on HPLC (peak c in Figure 1A). After 12 h, about 65−70% of the starting peptide was converted to the desired conjugate (observed mass = 952.47) (Figure 1A,B). These initial data indicate that this reaction is potentially compatible with large biomolecules.

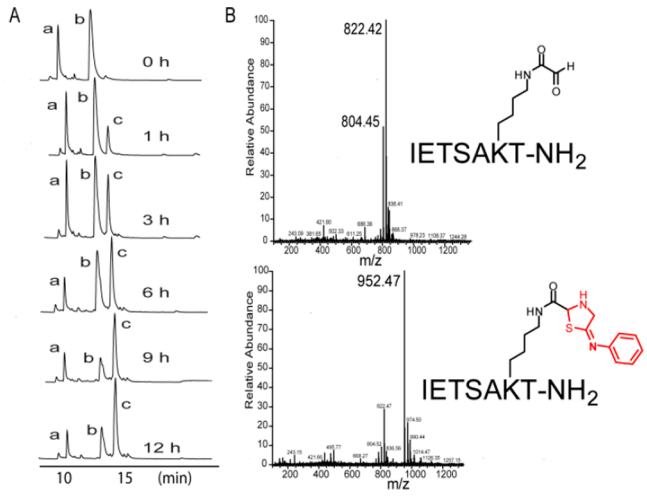


Figure 1. Characterization of the conjugation reaction of **1** with α-oxo aldehyde-containing peptide H-IETSAK(aldehyde)T-NH_2. (A) Analytical HPLC analysis of the reaction at different time slots. Peak a is H-IETSAK(aldehyde)T-NH_2, peak b is **1**, and peak c is the conjugated product. (B) Mass spectrometry analysis showing the mass of H-IETSAK(aldehyde)T-NH_2 (calcd mass: 803.40, found m/z: 804.45 $[M + H]^+$ and 822.42 $[M+H_2O+H]^+$) and the conjugated product (calcd mass: 951.45, found m/z: 952.47 $[M + H]^+$).

Next, we tested whether we could modify α-oxo aldehyde-containing proteins with the thioamide functionalized labeling reagents (Figure 2). Using the same $NaIO_4$ oxidation method,

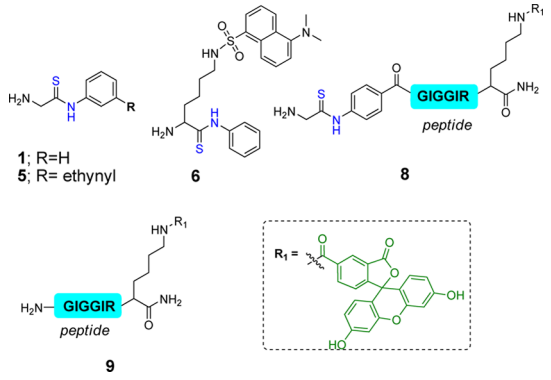


Figure 2. Structures of the thioamide-containing small compounds and peptide probes used in this study. Fluorescent probe **9** was used as the control.

the aldehyde functionality was introduced onto ubiquitin at its N-terminus via the oxidation of the first Ser residue (SI). Then ubiquitin-aldehyde **4** at 50 μM was incubated with 5 or 10 equiv of **1** in PBS (pH 7.2) at 4 and 24 °C, respectively. As seen by HPLC and MS analysis of the reaction mixture (Figure 3A,B),

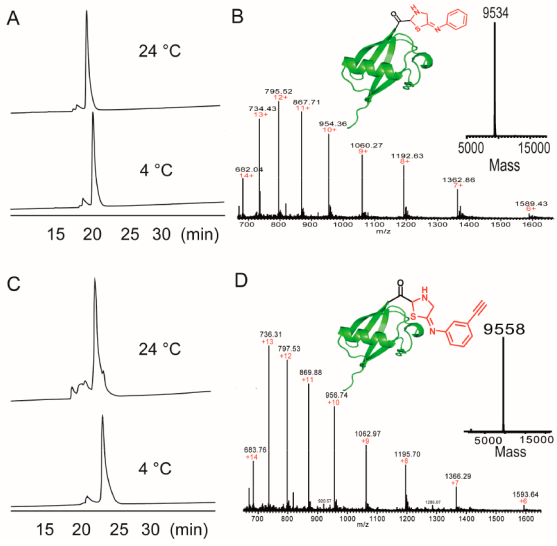


Figure 3. Characterization of the conjugation reaction between **1**, **5**, and ubiquitin-aldehyde **4**. (A) HPLC analysis of the bioconjugation of **1** with **4** for overnight at 4 and 24 °C, respectively. (B) Mass spectrometry analysis showing the mass of the conjugated product (calcd mass: 9531.9, found mass: 9534.0). (C) HPLC analysis of the bioconjugation of **5** with **4** for overnight at 4 and 24 °C, respectively. (D) Mass spectrometry analysis showing the conjugated product (calcd mass: 9555.9, found mass: 9558.0).

ubiquitin-aldehyde **4** was quantitatively conjugated with **1** after overnight reaction even at 4 °C. This result points to the potential of this method to label proteins that are sensitive to thermal stress. We also prepared a 2-aminoethanethioamide derivative **5**, which contains an alkyne group and performed the conjugation reaction with **4** under the same conditions. Again, it was shown that **4** was also quantitatively modified with **5** after overnight reaction (Figure 3C,D). Next, to label the protein with

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a fluorescent dye, we generated the dansyl-based fluorescent compound **6**. Initially, incubation with **6** resulted in very poor labeling of **4** in the aqueous PBS buffer (pH 7.2), likely due to the poor solubility of **6** in PBS. Therefore, we performed the reaction in the PBS/*t*BuOH (5:1), which has been used as a biocompatible solvent system for protein modification.[5] The reaction was kept at different temperatures (4, 24, and 37 °C) for overnight. Analytical HPLC and MS analysis show that **4** was more efficiently converted to the desired product at 37 °C with an estimated yield of 90% than at 4 °C (15%) and 24 °C (65%) (Figures 4A and 3B), indicating that increasing the solubility of **6** can help improve the conjugation efficiency.

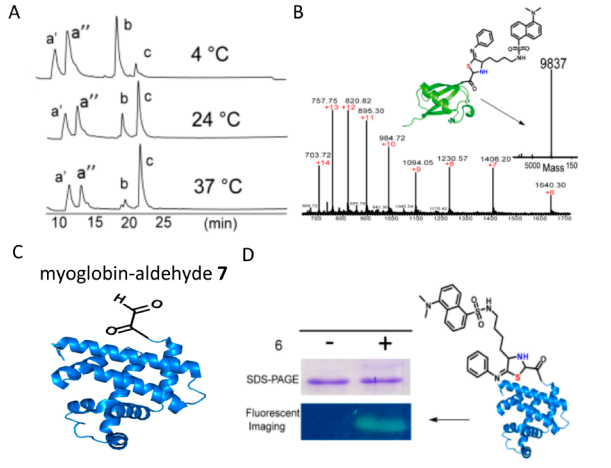

Figure 4. Characterization of the conjugation of **6** to ubiquitin-aldehyde **4** and myoglobin-aldehyde **7**. (A) HPLC analysis of the reaction between **6** (0.25 mM) and **4** (50 μM) at 4 °C, 24 and 37 °C for overnight, respectively. Peaks a′ and a′′ are **6**, peak b is **4**, peak c is the conjugated product. (B) Mass spectrometry analysis showing the conjugated product (calcd mass: 9836.3, found mass: 9837.0). (C) Structure of the myoglobin-aldehyde **7**. (D) Coomassie blue stained SDS-PAGE gel and fluorescent imaging of **7** (15 μM) before and after reacting with **6** (0.75 mM) for overnight at 37 °C. Fluorescent imaging was taken under UV 365 nm.

Myoglobin was also used as another model protein to further test this conjugation reaction. PLP (pyridoxal 5′-phosphate)-mediated transamination was used to generate the unique α-oxo aldehyde at the N-terminus of the protein.[6] Consistent with the previous reports,[5,6] the aldehyde can be quantitatively introduced into the myoglobin (Figures 4C and S4). Incubation of myoglobin-aldehyde **7** (15 μM) with **1** (0.75 mM, 50 equiv) for overnight afforded the conjugate in more than 95% yield at all tested temperatures (4, 24, or 37 °C) by LC-MS analysis (Figure S5). Compound **5** was also efficiently conjugated with **7** in the same conditions with an estimated yield of 60% at 4 °C, 70% at 24 °C, and 90% at 37 °C, respectively (Figure S6). While **6** was also conjugated with **7**, the overall yield was 40−45% even at 37 °C after overnight reaction. No significant product was observed at 4 and 24 °C, again due to poor solubility of **6** (Figures S7). Furthermore, we also demonstrated specific labeling of the phage-surface protein p8 after PLP-mediated transamination,[7] which introduced a ketone group, as confirmed by analytical HPLC, ESI-MS, and fluorescent gel analysis (Figures S8−S11). However, the labeling was significantly less efficient, likely because the ketone group in the α-oxopropanamide is much less reactive than the aldehyde in α-oxoacetamide. All these data indicate that the aldehyde−aminoethanethioamide condensation system is a novel bioorthogonal conjugation reaction for site-specific modification of proteins in their native states.

Specific labeling of live cell surfaces has proven valuable to manipulate cell fate and functions for basic and translational research with numerous applications in biotechnology and medicine.[8] Thus, we decided to demonstrate such a utility of our method for cell surface labeling and test how efficient it is. The aldehyde group was easily generated on the cell surface (Hela cell line) via the widely used $NaIO_4$ oxidation of the sialic acid residues in cell-surface glycans.[9] Two fluorescent probes **8** and **9** were prepared (Figure 2). Fluorescent probe **8** harbors the 2-aminoethanethioamide at the N-terminus of the peptide, while the control probe **9** does not (Figure S12−S13). In the labeling experiment, 20 μM **8** or **9** was added to the $NaIO_4$-treated and nontreated cells, respectively, and the cells were then incubated at 37 °C for 30 min. The probes were removed by washing the cells with PBS three times, and the treated cells were analyzed using confocal microscopy. Results show that only in the $NaIO_4$-oxidized cells treated with **8** was significant fluorescence observed on the rim of their membranes (Figure 5A). $NaIO_4$-

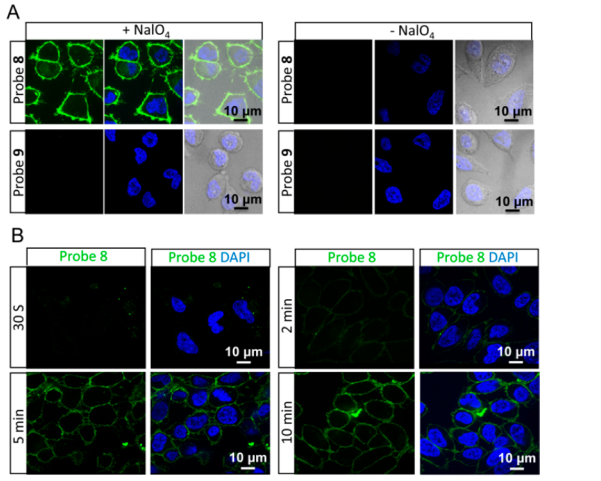

Figure 5. Labeling of cell surface using 2-aminoethanethioamide and aldehyde condensation reaction. (A) Confocal microscope images of cells labeled by probe **8** and **9** at 37 °C for 30 min. Nontreated cell by $NaIO_4$ was used as the control. (B) Confocal microscope images of cells labeled by probe **8** and **9** at 37 °C during 30 s to 10 min. Nucleus was stained with DAPI.

oxidized cells treated with **9** or nonoxidized cells did not generate any significant fluorescent signal (Figure 5A). We also performed the time course labeling of the cells from 30 s to 10 min. As seen from Figure 5B, the labeling of cells could be observed at as early as 5 min. These results further confirm the efficient nature of the aldehyde−aminoethane thioamide condensation reaction, which can be used to label living cell surfaces under mild conditions. Efficient cell labeling was also confirmed by the fluorescence-activated cell sorting (FACS) analysis (Figure S14). Fast labeling is usually required to study certain cellular events such as dynamic membrane trafficking or for specific receptor tracking. In our study, after 30 min of labeling, the cells were returned to culture medium at 37 °C for 1 and 2 h. Then the cells were fixed and subjected to confocal microscopy. It was shown that after 1 h, some portions of the labeled membrane were found inside the cells, presumably via internalization, which revealed a dynamic process of membrane

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component trafficking and turnover (Figure S15). Previously, an oxime ligation-based method used 10 mM aniline as the catalyst and a higher concentration (100 μM) of the labeling probe and required an incubation time of 90 min to achieve efficient cell labeling.[9] Our method does not need a catalyst and requires a lower concentration (20 μM) of the probes and shorter labeling time (30 min). These are desirable features of a biocompatible method to label, monitor, and track the molecules on the cell membranes.

In summary, we have shown that the condensation between 2-aminoethanethioamide and an aldehyde is highly chemoselective. It was successfully utilized to site-selectively label peptides, proteins, and living cells. The reaction proceeds smoothly in an aqueous buffer under mild conditions, uses very low reactant concentrations, and does not require any catalysts or reducing agents that might be toxic to living systems. The thiazolidin-5-imine linkage so-formed is stable at physiological and weakly basic pH, while hydrolyzable in acidic media (e.g., pH 4.5). Such a property of pH-dependent stability and reversibility would be useful for drug delivery purpose.[10] It is noteworthy that a related thiazolidine ring formed between the 1,2-aminothiol moiety of an N-terminal Cys residue and an aldehyde is not very stable under physiological pH but rather stable in slightly acidic pH.[11] As another favorable characteristic of this new method, the 2-aminoethanethioamide moiety can be easily installed in any compounds and is very stable in air. On the contrary, the 1,2-aminothiol moiety required for thiazolidine formation is very prone to oxidation in which the thiol is oxidized to disulfide.[12] Aldehyde is one of the most extensively utilized bioconjugation functionalities, and many chemical and enzymatic methods are available to introduce an aldehyde group into proteins at either a terminal or internal site.[6,13] Therefore, we anticipate that this new condensation reaction will be an attractive alternative to the currently used bioconjugation techniques for a wide range of applications in the future.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.orglett.8b03195.

Experimental procedures and characterization data (PDF)

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Notes

The authors declare no competing financial interest.

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