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. 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. 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. 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). Due to poor solubility of the two compounds in water, 1 was allowed to react with 2 in PBS/acetonitrile/tBuOH 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 (1H and 13C) and mass spectrometry (Figure S1-2 in the Supporting Information (SI)). 1H 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 in the SI). 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 and analyzed by NMR (1H and 13C) and mass spectrometry (Figure S1-2 in the Supporting Information (SI)). We find that 3 is stable in aqueous buffer at pH 4.5 for up to 48 h, at pH 7.2 for up to 24 h, and at pH 8.5 for up to 8 h. The remaining product 3 was then further used for bioconjugation studies.
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).

**Scheme 1.** Model reaction between 1 and α-oxo aldehyde 2 to yield the condensation product 3 and the proposed mechanism.

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-NH2, where X = α-oxo-aldehyde which was introduced via NaIO4 oxidation of the 1,2-aminoethanol moiety of a Ser residue (Scheme 2 and SI). The condensation reaction 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.

**Scheme 2.** Generation of an α-oxo aldehyde in a model peptide and its subsequent bioconjugation with 1.

Figure 1. Characterization of the conjugation reaction of 1 with α-oxo-aldehyde-containing peptide H-IETSAK(Aldehyde)T-NH2. (A) Analytical HPLC analysis of the reaction at different time slots. Peak a is H-IETSAK(Aldehyde)T-NH2, peak b is 1 and peak c is the conjugated product. (B) Mass spectrometry analysis showing the mass of H-IETSAK(Aldehyde)T-NH2 (calcd mass: 803.40, founded mass: 804.45 and 822.42 [M+H2O+H]+) and the conjugated product (calcd mass: 951.45, founded mass: 952.47).

Next, we tested whether we could modify α-oxo-aldehyde-containing proteins with the thioamide functionalized labeling reagents (Figure 2). Using the same NaIO4 oxidation method, 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 equivalents of 1 in PBS (pH 7.2) at 4 °C and 24 °C, respectively. As seen by LC-MS analysis of the reaction mixture (Figure 3A, 3B), 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 and 3D). Next, to label the protein with 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/BuOH (5:1), which has been used as a biocompatible solvent system for protein modification. 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 and 24 °C (Figure 4A and 4B), indicating that increasing the solubility of 6 can help improve the conjugation efficiency.

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. Consistent with the previous reports, the aldehyde can be quantitatively introduced into the myoglobin (Figure 5C and Figure S4 in the SI). Incubation of myoglobin-aldehyde 7 (15 μM) with 1 (0.75 mM, 50 equivalents) for overnight afforded the conjugate in more than 95% yield at all tested temperatures.
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 °C and 24 °C, respectively. (B) Mass spectrometry analysis showing the mass of the conjugated product (calcd mass: 9531.88, found mass: 9534). (C) HPLC analysis of the bioconjugation of 5 with 4 for overnight at 4 °C and 24 °C, respectively. (D) Mass spectrometry analysis showing the conjugated product (calcd mass: 9555.9, founded mass: 9558).

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 °C and 37 °C for overnight, respectively. Peak 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: 9836.29, founded mass: 9837). (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.

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. Non-treated cell by NaIO4 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.

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. 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 NaIO4 oxidation of the sialic acid residues in cell-surface glycans. 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 S12-13 in the SI). In the labeling experiment, 20 μM 8 or 9 was added to the NaIO4-treated and non-treated cells, respectively, and the cells were then incubated at 37 °C for 30 min. The probes were removed by washing the cells with PBS for 3 times and the treated cells were analysed using confocal microscopy. Results show that only in the NaIO4-oxidized cells treated with 8, was significant fluorescence observed on the rim of their membranes (Figure 5A). NaIO4-oxidized cells treated with 9 or non-oxidized cells did not generate any significant fluorescent signal (Figure 5A). We also performed the time course labeling of the cells from 30 sec 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 Fluores-
cience-Activated Cell Sorting (FACS) analysis (Figure S14 in the SI). 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, revealing a dynamic process of membrane component trafficking and turnover (Figure S15 in the SI). 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 in order to achieve efficient cell labeling. 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 the molecules on the cell membranes. It is noteworthy that a related thiazolidine ring stability and reversibility would be useful for drug delivery acidic media (e.g., pH 4.5). Such a property of pH-dependent catalysts or reducing agents which might be toxic to living smoothly in an aqueous buffer under mild conditions, uses physiological and weakly basic pH, while hydrolysable in

ASSOCIATED CONTENT

Supporting Information

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

Experimental procedures and characterization data (PDF)

AUTHOR INFORMATION

Corresponding Author

* E-mail: cfliu@ntu.edu.sg

ORCID

Peter C. Dedon: 0000-0003-0011-3067
Chuan-Fa Liu: 0000-0001-7433-2081

Notes

The authors declare no competing financial interest.

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