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Catalyst free hydrazone ligation for protein labeling and modification using electron-deficient benzaldehyde reagents

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Bioorthogonal reactions have emerged as valuable tools for site-specific protein labeling and modification *in vitro* and *in vivo*. The hydrazone and oxime ligation has recently attracted considerable attention for the widly applications in the conjugation of biomolecules. However, this kind of reaction has been suffered from slow kinetics under physiological conditions and the toxicity or complication of the reaction system due to catalysts. In this work we have developed an electron-deficient benzaldehyde reagent, which can be easily equipped with various types of bio-functional molecules for catalyst-free hydrazone ligation. The reagents can be equipped with not only small molecules such as fluorescence dyes or drugs, but also macromolecules like PEG. They can be precisely ligated to the C-terminus of the proteins by efficient hydrazone reaction at neutral pH and room temperature. The new regeant based catalyst-free hydrazone ligation provides a practical approach for the site specific modification of proteins.

Introduction

Site-specific chemical modification of protein is a critical aspect of chemical biology and biomaterials science.[1] The site-specificity and mild reaction conditions are the keys to achieve an accurate modification without interfering with the structure and function of the protein. During the past decade, various types of bioconjugation reactions have been developed.[2] As an alternative, imine-based hydrazone and oxime ligation have recently attracted much attention in this field since it can proceed under mild conditions.[3] Additionally, the reversibility of the hydrazone and oxime conjugation is a powerful feature for the controlled release. Hence, the hydrazone and oxime ligation has wide applications in the conjugation of biomolecules.[4] However, this kind of reaction has been suffered from slow kinetics under physiological conditions.[5] Many scientists demonstrated that aniline and some more efficient molecules can be used as catalysts in these reactions.[6] However, these catalysts complicate the reaction system, although they are efficient.[7]

To address this issue, some groups progressed in the study of catalyst-free imine formation.[7a, 8] Based on structure optimization, some fast-reacting substrates have been designed. These catalyst-free imine formation can more efficiently modulate the peptides, proteins and nucleic acids with fluorescent molecules (Fig. 1a). Although promising results have been derived, this catalyst-free concept has not been applied to protein modification with more valuable molecules, such as PEG and drugs.[9] Therefore, discovering catalyst-free ligation methods for more complex molecules will significantly improve the utility and broaden the scope of this valuable bioorthogonal reaction.

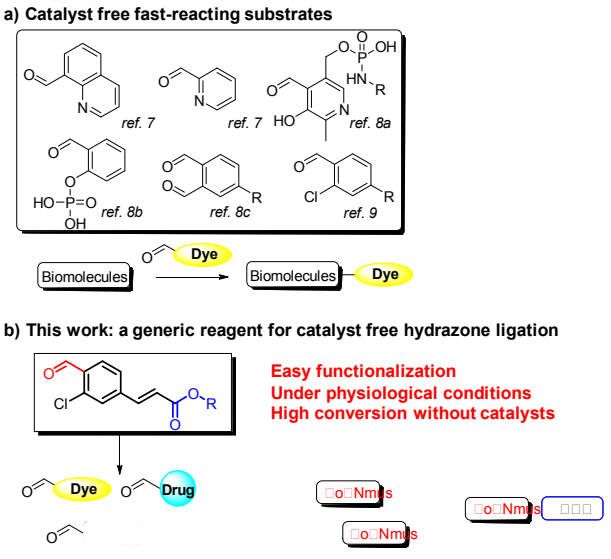

Figure 1 (a) Reported catalyst free fast-reacting substrates and applications; (b) A generic reagent for catalyst free hydrazone ligation.

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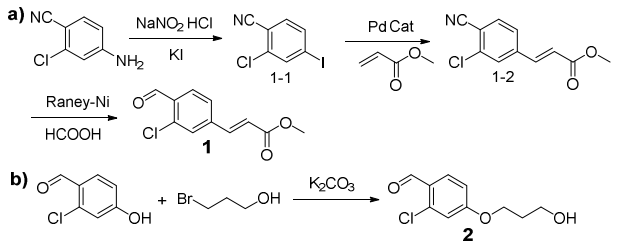
Here, we demonstrated a simple catalyst-free hydrazone ligation strategy for protein modification by using electron-deficient benzaldehyde reagents. This method enabled preparation of modified protein with different biofunctional molecules. The Key point of this method was the development of a generic reagent, which presented the application of catalyst-free PEGylation of protein. Moreover, protein modifications with fluorescent molecular and drug were also performed (Fig. 1b).

Results and discussion

Because an effective bioconjugation must have the ability to attach various synthetic groups, we began our study by designing the high reactive activity reagents with general handles. In our previous study, we reported that 2-chlorobenzaldehyde can be incorporated into protein substrates by hydrazone formation.[9] Moreover, inspired by previous reports that electron-withdrawing groups can increase the reactivity of aldehyde or ketone in imine formation, our group explored the potential of the electronic effects on 2-chlorobenzaldehyde based catalyst-free hydrazone formation.[7a] Therefore, we designed a compound 1 with electron-withdrawing group. For comparison, we also designed compound 2 with electron-donating group. As shown in Scheme 1, compound 1 was synthesized in three steps from 3-chloro-4-isocyanoaniline. It has a terminal methyl protected carboxyl group for later functionalizations. Compound 2 was synthesized through a one-step reaction and has a hydroxyl group as a handle.



Scheme 1 Synthetic routes of two reagents. (a) Synthetic route of benzaldehyde with electron-withdrawing group; (b) Synthetic route of benzaldehyde with electron-donating group.

Our works began with the screening of the reactivity of aldehydes by using a model peptide LYRAF-$NHNH_2$. We also selected 2-chlorobenzaldehyde (3) for this testing as comparison (Fig. 2 and Fig. S1). The reactions were run with 30 μM peptide, 37.5 μM aldehyde. Then the mixture was stirred under neutral pH and 25 °C for about 0.5 h, followed by the termination of conversion by RP-HPLC. Remarkably, the reaction was complete (95% yield) using compound 1 in 0.5 h (Fig. 2b). It is worth noting that although benzaldehyde with electron-donating group is theoretically not suitable for this condensation reaction, compound 2 also exhibits higher reactivity than 2-chlorobenzaldehyde (Fig. 2c and 2d). Moreover, Ubiquitin hydrazine also showed similar results (Fig. S2).

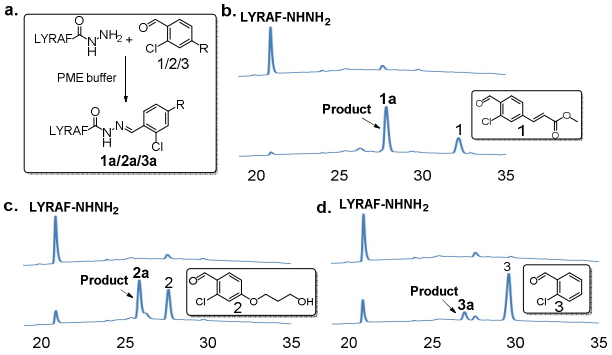


Figure 2. Conjugates between peptide hydrazides and different aldehydes. (a) Peptides with the sequence Leu-Tyr-Arg-Ala-Phe-$NHNH_2$ was tested for reactivity with different aldehydes; (b) peptide hydrazides reacted with compound 1; (c) peptide hydrazides reacted with compound 2; (d) peptide hydrazides reacted with compound 3. The following are detailed HPLC conditions, solution A was 0.1% TFA in water, and solution B was 0.1% TFA in MeCN. Gradient: A linear gradient of 1% to 90% B over 35 min.

Next, we investigated the general utility of this optimal reagent 1 by testing the conversion yield of model peptides with different C-terminal amino acids (H-Leu-Tyr-Arg-Ala-X-$NHNH_2$). In detail, peptide hydrazides and aldehydes (30 μM and 200 μM in final concentration) were added in aqueous PME buffer (100 mM PIPES, 1 mM $MgSO_4$, 2 mM EGTA at pH 7.0) with 20% alcohol. As shown in Table 1, the yield of hydrazone formation for each peptide was relatively high within 0.5 h. The yield didn't increased significantly after a longer reaction time. An unusual phenomenon was glycine lead to low yield in contrast to other sterically more demanding residue. And phenylalanine and tryptophan showed the highest yield.

Table 1 Conjugates between reagent 1 and model peptide-$NHNH_2$ with different C-terminal residues

Entry	X	HPLC yield [%][a]
1	Gly	65
2	Phe	95
3	Leu	71
4	Pro	76
5	Arg	69
6	Trp	94

[a] 30 μM peptide hydrazide and 200 μM aldehyde

To develop a general and effective bioconjugation method, it is important that various functional groups can be readily attached to the benzaldehyde reagent. Many biofunctional molecules, which bear a free hydroxyl group handle or can be equipped with a hydroxyl group through simple reaction, are

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commercially available. It will allow the easy access to functionalized reagents through esterification of the corresponding carboxylic acid intermediate.

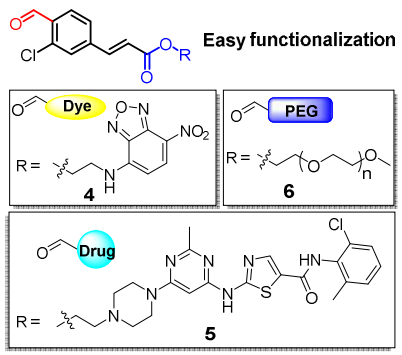

Figure 3. The functionalization of reagent 1. 4, benzaldehyde derivative with dye; 5, benzaldehyde derivative with drug; 6, benzaldehyde derivative with polyethylene glycol.

For labeling of a dye, fluorescent benzaldehyde derivative 4 was synthesized in two steps from 4-Chloro-7-nitrobenzofurazan (ex=465 nm and em=539 nm) (Fig. 3).[10] Then the coupling reaction was performed by adding 30 μM Ub with a C-terminal hydrazine, 200 μM compound 4, PME buffer (pH 7.0) in a final volume of 1mL and kept at room temperature for 0.5 h.[13] Next, to confirm the labeling of protein, the reaction mixture was examined by SDS-PAGE and in-gel fluorescence analysis. Unfortunately, no fluorescence was observed and the possible reason was that compound 4 was too hydrophobic to react with the protein. Thus we added 30% DMF in the reaction mixture to improve the solubility of compound 4. Then the fluorescence was detected (Fig. 4d, line 2). MS analysis of the product also showed the labelled protein (Fig. 4b). A negative control using the protein without the C-terminal hydrazine showed no fluorescence (Fig. 4d, line 1). Taken together, this data not only demonstrated that probe fluorescence was retained after functionalization of the protein with the benzaldehyde reagent, but also proved that the reaction had a precise selectivity.

To further exemplify the utility of our method for bioconjugation, we decided to introduce a more complex molecule Dasatinib.[11] Dasatinib is a chemotherapy medication used to treat certain cases of chronic myelogenous leukemia (CML) and acute lymphoblastic leukemia. The derivative 5 which contains Dasatinib was synthesized through one step esterification. With this drug containing derivative in hand, we carried out the reaction at the same condition with compound 4. MS analysis showed the correct drug-ligated protein (Fig. 4c)

Then we aimed to realize the conjugation between protein hydrazide and macromolecule through catalyst-free hydrazone ligation based on this new type of benzaldehyde reagent. Polyethylene glycol (PEG) is the one of the most commonly used polymers in the wide field of medicinal chemistry.[12] Site-specific attachment of PEG to protein has also gained particular interest. The attachment of PEG chain to protein has been shown with good biocompatibility, non-immunogenity and resistance to protein degradation. To investigate whether benzaldehyde reagent can be used in this important area, we synthesized the PEG functionalized benzaldehyde reagent 6 by using Methoxypoly (ethylene glycol) (average MW=2000 Da) (Fig. 3). Then we tested the ligation between reagent 6 and Ub-$NHNH_2$. Ub-$NHNH_2$ (30 μM) in its native tertiary structure was reacted with 5 folds of compound 6 in PME buffer. After 0.5 h, conversion was determined using HPLC and SDS-PAGE (Fig. 4e, and Fig. 4d, line 3). Surprisingly, the reaction showed excellent level of conversion (Conv. 96%). Maldi-TOF-MS analysis showed that the molecular weight of product was around 10750 Da (Fig. 4f), which further confirm the corrected conjugation between PEG and protein.

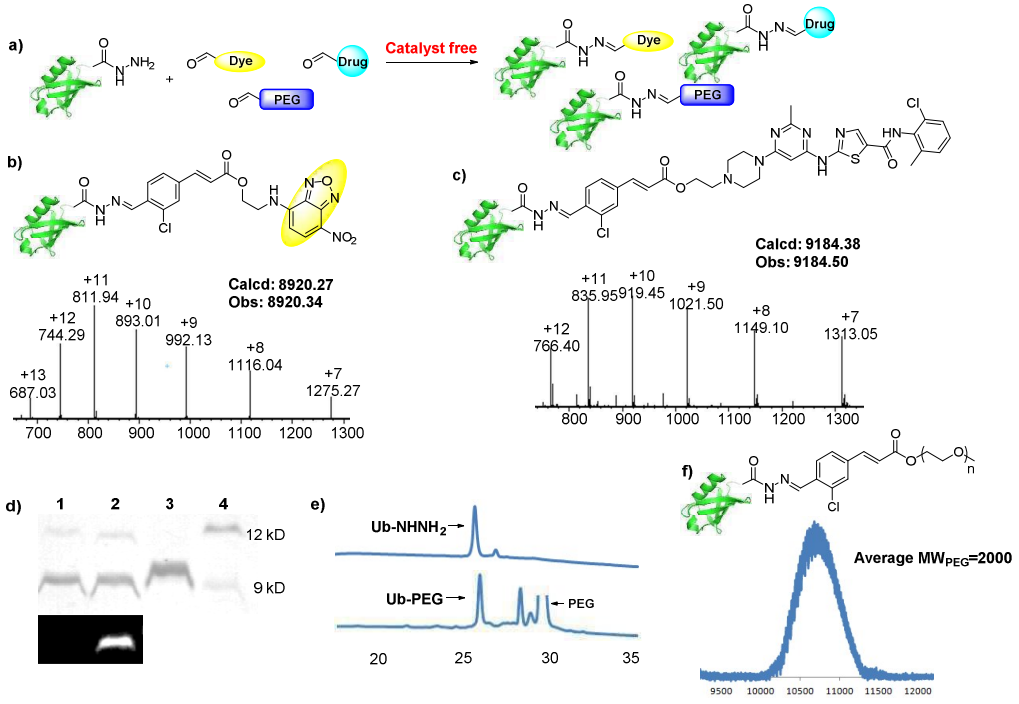

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Figure 4. Protein modification with different functionalized reagents. (a) Catalyst free hydrazone ligation between ubiquitin and dye, drug and PEG; (b) ESI-MS analysis of fluorescent ligated ubiquitin; (c) ESI-MS analysis of drug ligated ubiquitin; (d) SDS-PAGE analysis of fluorescent and PEG labelled ubiquitin, line 1: protein without the C-terminal hydrazine, line 2: fluorescent ligated ubiquitin, line 3: PEG labelled ubiquitin, line 4: marker; (e) HPLC analysis of PEG labelled ubiquitin; (e) Maldi-Tof-MS analysis of PEG labelled ubiquitin.

Conclusions

In summary, we have developed a generic electron-deficient benzaldehyde reagent for catalyst free hydrazone ligation at neutral pH. In contrast, previously used 2-chlorobenzaldehyde can only achieve labeling of proteins with simple small molecules by moderate efficiency, and electron-deficient benzaldehyde reagents can efficiently equip large molecules, possibly due to electron-withdrawing groups. This reagent has benzaldehyde handle for conjugation and a terminal methyl protected carboxyl group for functionalize bioactive molecules such as fluorescence dye, drug molecule and PEG. Through catalyst free hydrazone ligation, we achieve efficient bioconjugation between target protein and benzaldehyde reagent. To the best of our knowledge, this is the first time to realize macromolecule modification of protein by using catalyst free hydrazone ligation. Taken together, this reagent provides a powerful method for access to protein modification methodologies.

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Conflicts of interest

There are no conflicts to declare.

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