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A chemical covalent tactic for bio-thiol sensing and protein labeling agent design†

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Discovering novel chemical reactions is important for bioanalysis. Herein, we report a tactic for bio-thiol sensing and protein labeling agent design by the installation of a sulfoxide group onto the skeleton of various fluorophores, and powerfully validate its abilities, which may shed light on the development of specific protein tags to give insight into their biological functions.

Selective chemical covalent labeling of protein has become an essential tactic for probing protein functions *in vitro* and/or in living systems. Hence discovering novel chemical reactions under mild conditions is a priority for developing novel tactics of researching cellular biomolecules. Many efficient methods for chemical protein labeling by bioorthogonal reactions have been developed and summarized in some excellent reviews.[1–7] For redox systems, the biological thiols, low-molecular-weight thiols and protein thiols play crucially important roles in maintaining protein structures and functions, regulating redox balance and cell signaling. They are extremely susceptible to reactive oxygen species (ROS) and easily resulted in a range of oxidative sulfur post-translational modifications including diverse mainstream intermediates, such as sulfenic acids, sulfinic acids, sulfonic acids, sulfonamides, persulfides, and various disulfides including intramolecular or intermolecular disulfide bridges, *etc.*[8–10] Therefore, low-molecular-weight thiol sensing and protein thiol labeling tremendously help to understand their biological roles of redox regulation and signaling. And most importantly, their roles in these processes are not clearly understood and not given suitable elucidations due to lack of powerful agents for site-specific protein labeling. Small molecular fluorescent probes have proven to be invaluable tools for thiol sensing[11–17] and protein labeling,[18–26] showing excellent advantages on studying localizations, distributions, movements, interactions, functions and microenvironments of small biological molecules and proteins inside live cells over traditional methods, such as simple manipulation, real-time monitoring, and high temporal and spatial resolution. Hence, novel tactics for designing protein thiol agents are always urgently needed to expand profound understanding of protein cell biology.

Although thiol labeling/blocking reagents available for protein thiol/vicinal thiol sensing or labeling, and redox states study based on the fundamental skeleton of response groups which are listed in Fig. 1A but not all,[11,12,26–35] there remain drawbacks of this or that kind during their use. It is a great challenge how to exploit a novel strategy for designing protein thiol sensing or labeling agents with highly selective and fast response time towards specific sites of the target protein. Recently, what is particularly noteworthy is that an electron-withdrawing substituent, such as a nitro, sulfoxide or sulfone

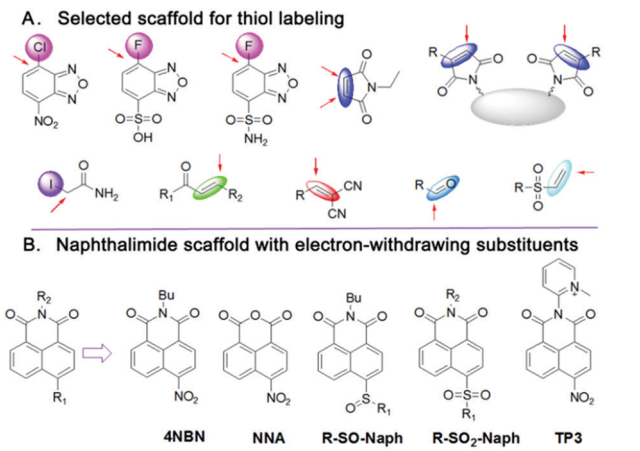

Fig. 1 The labeling agent structures of previous work.

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group, at the 4-position of the 1,8-naphthalimide (Naph) scaffold as a leaving group has been researched for thiol sensing (Fig. 1B).[36–40] Unfortunately, the main drawback of this kind of scaffold is that the reaction products of these Naph-based compounds and thiols failed to give stable fluorescence signals and gradually quenched during the imaging.

In the past several years, we have been always making endeavors to develop fluorescent toolkits for various species to probe redox events inside live cells.[41–45] Recently, we have developed a small library for the designing of Msr probes by the installation of a methyl sulfoxide moiety on various fluorophore scaffolds (Fig. 2).[42] During screening of probes, we discovered that some compounds showed a positive fluorescence response to thiols (compound **1–3**, **14**, **17** in Fig. 2 & Table S1, ESI†), which inspired us to explore the mechanism underlying this phenomenon and gave birth to the present strategy for protein thiol labeling. In consideration of previous investigations and experimental results, compounds **1–3** were excluded firstly due to their aforementioned disadvantages (Fig. S1, ESI†). In the comparisons of compounds **14** and **17**, compound **17** (named as C-SOMe) exhibited more obvious fluorescence enhancement and was picked up for the follow-up thiol sensing and protein thiol labeling studies. Encouragingly, the C-SOMe shows high selectivity and fast response time toward thiol at a micromole level in several minutes, which greatly elevated the performances of thiol sensing and labeling. The reaction mechanism and protein labeling are firmly confirmed using diverse means of H NMR, MS spectra, HPLC analysis, imaging and western blot.

As depicted in Scheme S1 (ESI†), C-SOMe was easily obtained in two steps with a satisfactory yield at 50.2% in an extremely simple manner.[42] Encouraged by the initial results, we examined the reaction capacity of C-SOMe towards bio-thiols firstly in PBS buffer (10 mM, pH = 7.40) at 37 °C. As shown in Fig. 3A–C, the emission peak shifted from 550 nm to 510 nm when C-SOMe was incubated with thiols (GSH, Cys, and Hcy). The fluorescence intensity enhanced promptly with increasing time and then saturated. Interestingly, the fluorescent spectra changes exhibited a ratiometric property with excitation wavelength at 428 nm, which can provide a built-in correction for environmental effects, showing great advantages over other probes that are merely based on fluorescence increases or decreases.[46–48] Meanwhile, the fluorescence of the solution becomes bright green (Fig. 3D). UV-vis absorption spectra of C-SOMe with thiols were shown in Fig. S2 (ESI†). Fluorescence response of C-SOMe with various concentrations of bio-thiols were recorded and shown in Fig. S3A–C (ESI†). The detection limit of C-SOMe for Cys, GSH, and Hcy was determined to be 0.39 μM, 0.74 μM, and 1.38 μM by the reported method ($3\sigma/k$), respectively.[49,50] In Fig. 3E, the kinetic analysis was manipulated by addition of GSH, Cys and Hcy (100 equiv.) to C-SOMe (10 μM) and the fluorescence reached the maximum within 40 s. The pseudo-first-order rate constants were determined (Fig. S4, ESI†) and are summarized in Table S2 (ESI†). In addition, the selectivity is an important criterion for judging the performance of the labeling agent. In Fig. 3F, the fluorescence of C-SOMe is merely activated by bio-thiols, and other analytes elicited negligible fluorescent signals, which is due to the strong

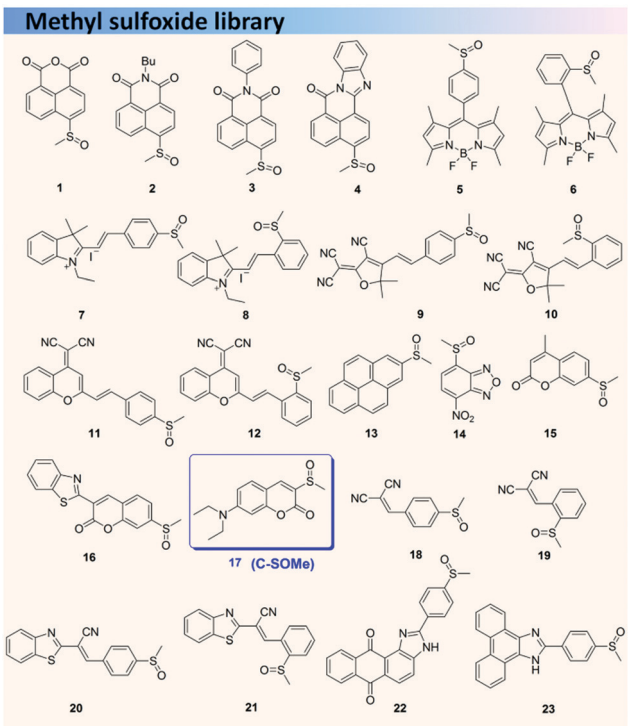

Fig. 2 The library of compounds containing a sulfoxide group.

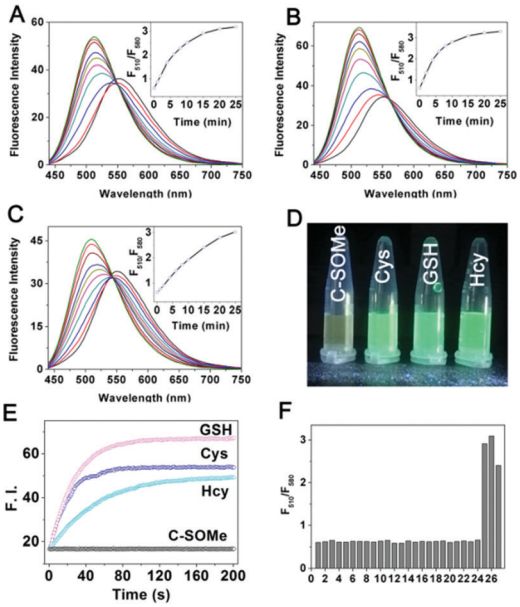

Fig. 3 Spectral studies of C-SOMe reacted with bio-thiols. Time-dependent fluorescent response of C-SOMe (10 μM) in the presence of 5 equiv. of Cys (A), GSH (B), and Hcy (C). Inset: Fluorescence change of F_{510}/F_{580}. (Fluorescence intensity with a large change at 580 nm), (λ_{ex} = 428 nm). (D) Fluorescence of C-SOMe towards bio-thiols under a 365 nm lamp. (E) Kinetic profiles of C-SOMe (10 μM) with 100 equiv. of Cys, Hcy, and GSH at room temperature. (F) Selectivity of C-SOMe towards different analytes for 15 min, 1 represents free C-SOMe (10 μM), 2–24 (1 mM) represent analytes including Phe, Pro, Arg, Thr, Lys, Ala, Glu, Gly, Ser, Val, Ile, Leu, Met, Trp, Asn, Tyr, Asp, Fe^{2+}, Fe^{3+}, H_2O_2, ClO^-, Ascorbic acid, and Na_2S, and 24–27 represent 50 μM Cys, GSH, and Hcy.

nucleophilicity of the thiol group and the positive center of a carbon atom caused by strong electron-withdrawing of the sulfoxide group. All these solid results revealed that the C-SOMe could readily react with these thiols under mild conditions with high sensitivity and selectivity.

In order to confirm that C-SOMe could be quantitatively and exclusively converted to its nucleophilic substituted compound without reduced compound, we interrogated the reaction mechanism of C-SOMe with thiol using NMR spectra and HPLC analysis. We selected Cys as a model compound to study the reaction mechanism initially. However, the formed adduct with poor solubility could hardly be characterized further. Therefore, another model compound, 2-mercaptoethanol, was used for the proposed mechanism studies (Fig. 4A). As shown in Fig. 4B, the fluorescent spectral changes are well consistent with that of C-SOMe and bio-thiols (Fig. 3). Notably, the response time is longer than that of GSH and Cys, and almost matching that of Hcy. This is not surprising and can be explained by their values of pK_a (Cys, 8.3; GSH, 8.8; Hcy, 10.0; 2-mercaptoethanol, 9.6). The detailed synthesis procedure of the formed adduct Coum-S is given in the ESI† and characterized using NMR (Fig. 4C and Fig. S5–S7, ESI†). Obviously, the proton signal at 2.88 ppm disappeared, while two new peaks at 3.98 ppm and 3.24 ppm appeared, which belong to the methylene of 2-mercaptoethanol. In the meantime, the aromatic protons of the coumarin skeleton shifted downfield or upfield, respectively (Fig. 4C and Fig. S9, ESI†). Subsequently, the MS spectrum was recorded with a peak at 294.12 ($[M + H]^+$) (Fig. S8, ESI†). All these proton signal changes and the MS spectrum clearly demonstrated that the proposed mechanism is reasonable. In addition, this reaction process is monitored using HPLC (Fig. 4D and Fig. S7, ESI†). In Fig. 4D, we monitored the conversion of C-SOMe to its adduct Coum-S by incubating it with 2-mercaptoethanol in PBS buffer at different time points (5 and 40 min). C-SOMe is almost converted to Coum-S at 40 min, which well meets with the results of fluorescence spectra. All the results further firmly confirmed that C-SOMe could react with bio-thiol based on the proposed reaction mechanism and be quantitatively converted to its nucleophilic substituted compound under mild conditions.

To verify that C-SOMe can be elicited by endogenous thiols, we performed fluorescence imaging for visualization of this process. As shown in Fig. 5, a bright blue fluorescence was observed after the Hep G2 cells were incubated with C-SOMe for 5 min (according to the principle of optical imaging fluorescence microscope, under the light source excitation can only produce blue fluorescence imaging signal, which mainly refers to the equipment of microscopy and fluorescence property of the probe).[40,43,45] Before treatment of the cells with C-SOMe, the cells were pretreated with *N*-ethylmaleimide (NEM, a thiol blocking reagent, 50 μM) for 0.5 h, and the fluorescence signal was almost completely inhibited. Colocalization experiments were carried out to elucidate whether the probe could stain whole cells (Fig. S10, ESI†). Bovine serum albumin (BSA), a simple protein, containing one free sulfhydryl group and 17 disulfides, is commonly used as a model protein for researching protein thiol labeling.[40,45] So we selected BSA as a model to examine the ability of C-SOMe in protein labeling. Before incubation with C-SOMe or NEM, BSA was treated with 10% SDS at 37 °C for 30 min. In Fig. S11 (ESI†), only incubation of BSA with C-SOMe induced a brilliant fluorescence band on the gel. The fluorescent signal was not observed on the gel when protein thiols were blocked by pretreatment with *N*-ethylmaleimide. The results of imaging and BSA labeling suggested the ability of C-SOMe for thiol sensing and specific targeting of sulfhydryl groups in the protein.

Finally, we have compared C-SOMe with previous agents, and discussion was given in supporting information (Part 5, ESI†).

In summary, novel tactics for designing protein thiol agents are extremely urgently required to meet needs in the field of cell biology, clinical medicine, and others. In this work, through

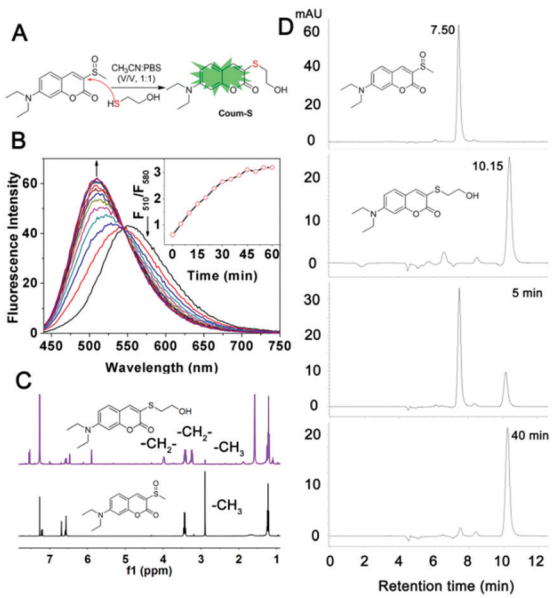

Fig. 4 (A) Proposed mechanism of C-SOMe reacted with thiol by using 2-mercaptoethanol. (B) Time course of fluorescence changes of C-SOMe (10 μM) towards 2-mercaptoethanol (50 μM). Inset: Fluorescence change of F_{510}/F_{580}. (λ_{ex} = 428 nm). (C) NMR spectra of C-SOMe and its adduct Coum-S. (D) HPLC analysis of C-SOMe with 2-mercaptoethanol.

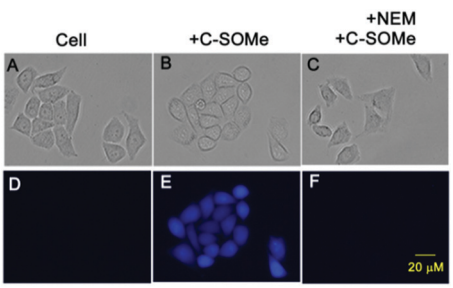

Fig. 5 Validation of C-SOMe is efficient for thiol inside Hep G2 live cells by fluorescence microscopy. (A and D) Only Hep G2 cells. (B and E) Hep G2 cells treated with C-SOMe (10 μM) for 5 min. (C and F) Hep G2 cells incubated with NEM (50 μM) for 30 min followed by further treatment with C-SOMe (10 μM) for 5 min (blue channel:λ_{em}: 480–580 nm).

systematically investigating methods for thiol labeling and combining previous studies on this aspect, we proposed an effective tactic for designing thiol labeling agents bearing a sulfoxide group, which exhibits brilliant performances of high sensitivity, selectivity and quantitative conversion. The capabilities of the agent are well demonstrated using fluorescent, NMR, MS spectra and HPLC analysis. We further validated its powerful abilities in thiol sensing and labeling using fluorescence microscopy and a BSA model. This tactic will provide a potential guideline for designing novel labeling agents with more brilliant performance.

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Conflicts of interest

There are no conflicts to declare.

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