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Fluorescent Probes

Active-Site-Matched Fluorescent Probes for Rapid and Direct Detection of Vicinal-Sulfydryl-Containing Peptides/Proteins in Living Cells

Xiaohong Pan,[a, b] Ziye Liang,[a] Jing Li,[a] Shanshan Wang,[a] Fanpeng Kong,[a] Kehua Xu,*[a] and Bo Tang*[a]

Abstract: Vicinal-sulfydryl-containing peptides/proteins (VSPPs) play a crucial role in human pathologies. Fluorescent probes that are capable of detecting intracellular VSPPs in vivo would be useful tools to explore the mechanisms of some diseases. In this study, by regulating the spatial separation of two maleimide groups in a fluorescent dye to match that of two active cysteine residues contained in the conserved amino acid sequence (–CGPC–) of human thioredoxin, two active-site-matched fluorescent probes, *o*-Dm-Ac and *m*-Dm-Ac, were developed for real-time imaging of VSPPs in living cells. As a result, the two probes can rapidly respond to small peptide models and reduced proteins, such as WCGPCK (W-6), WCGGPCK (W-7), and WCGGGPCK (W-8), reduced bovine serum albumin (rBSA), and reduced thioredoxin (rTrx). Moreover, *o*-Dm-Ac displays a higher binding sensitivity with the above-mentioned peptides and proteins, especially with W-7 and rTrx. Furthermore, *o*-Dm-Ac was successfully used to rapidly and directly detect VSPPs both in vitro and in living cells. Thus, a novel probe-design strategy was proposed and the synthesized probe applied successfully in imaging of target proteins in situ.

Introduction

Intracellular protein thiols play an essential role in maintaining redox homeostasis by regulating the redox status between reduced sulfhydryl residues (−SH) and oxidized disulfide linkages (−S−S−).[1,2] Vicinal-sulfydryl-containing proteins are considered to be highly effective in regulating the redox environment of internal cellular compartments under normal conditions.[3,4] However, under oxidative stress, the vicinal-sulfydryl-containing proteins are overoxidized by reactive oxygen species[5,6] and form an excess of disulfide-bonded proteins that may result in cellular dysfunction and then various diseases, including cancer,[7] diabetes mellitus,[8] and stroke,[9] as well as many neurological disorders such as Alzheimer's and Parkinson's diseases.[10–12] It is assumed that monitoring the concentration of VSPPs in living organisms may be of help in the early diagnosis and prevention of such diseases. Therefore, the development of rapid, selective, and sensitive detection methods for the thiol protein levels in biological systems is of significant importance.

Over the past few years, a number of detection techniques for protein thiols have been developed. Among the various analytical methods, fluorescence-based detection is more attractive due to advantages such as good selectivity, highly sensitivity, short reaction time, and potential application for in vivo imaging.[13–15] For this reason, a variety of protein-thiol-sensitive fluorescent probes have been developed.[16–19] However, all of these approaches to identify protein thiols rely on monitoring thiol groups indirectly by labeling of target peptide sequences, and the target peptides were exogenously introduced into the target proteins in living cells. The structure of endogenous thiol proteins in cells is complex, and most of them contain multiple thiol groups that are close to each other in space and play a key role in redox homeostasis.[20–22] Therefore, a design strategy for selective and direct detection probes for endogenous thiol proteins in living cells is in high demand. Zhu and co-workers selected naphthalimide as fluorophore and cyclic dithiaarsanes as binding group to design and synthesize a selective fluorescent probe for the detection of dithiol proteins in living cells.[23] Despite this progress, novel fluorescent probes with rapid response, low background, and other superior properties, such as easy synthesis, good cell permeability, high sensitivity, and high selectivity, are still lacking for direct detection of endogenous thiol proteins.

[a] Dr. X. Pan, Z. Liang, J. Li, S. Wang, Dr. F. Kong, Prof. K. Xu, Prof. B. Tang
College of Chemistry, Chemical Engineering and Materials Science
Collaborative Innovation Center of Functionalized Probes for Chemical Imaging in Universities of Shandong
Key Laboratory of Molecular and Nano Probes, Ministry of Education
Shandong Provincial Key Laboratory of Clean Production of Fine Chemicals
Shandong Normal University, Jinan, Shandong, 250014 (P.R. China)
E-mail: xukehua@sdnu.edu.cn
tangb@sdnu.edu.cn

[b] Dr. X. Pan
Department of Pharmaceutical Sciences
Binzhou Medical University, Yantai, Shandong, 264003 (P.R. China)

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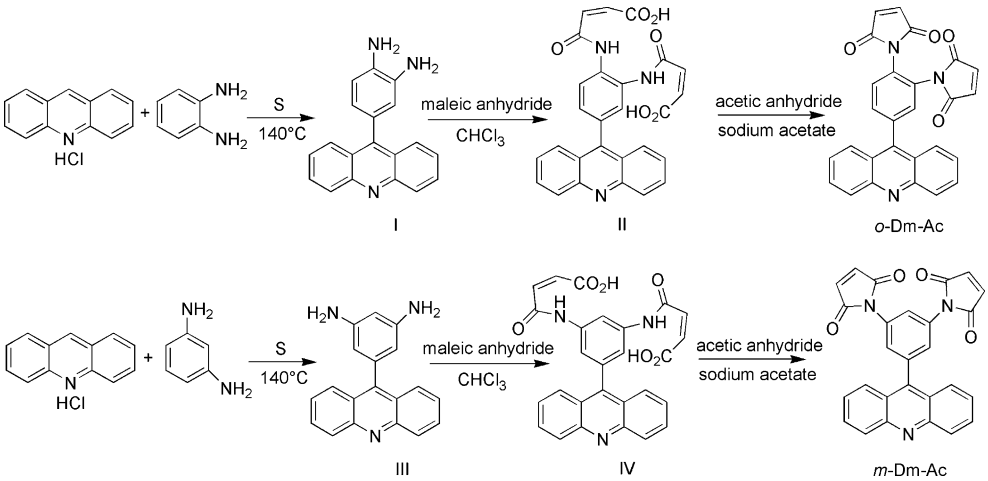

Scheme 1. Synthesis of *o*-Dm-Ac and *m*-Dm-Ac.

In this study, two active-site-matched fluorescent probes, *o*-Dm-Ac and *m*-Dm-Ac, were developed (Scheme 1) for the rapid direct detection and visualization of VSPPs both in vitro and in living cells. The two probes were designed by binding two maleimide groups, separated by different distances, as a pair of reactive groups selective to VSPPs, to an acridine dye that acts as a fluorophore. As small peptide models, WCGPCK (W-6), WCGGPCK (W-7), and WCGGGPCK (W-8) were designed and used to test the properties of the probes. Both of the probes can rapidly and selectively respond to these peptide models. Besides, *o*-Dm-Ac exhibits higher sensitivity than *m*-Dm-Ac, especially to W-7, and a high selectivity for vicinal-sulfydryl-containing proteins (e.g., rBSA and rTrx) over other proteins. Furthermore, *o*-Dm-Ac was successfully used for real-time imaging of VSPPs both in vitro and in living cells.

Results and Discussion

Design and synthesis of *o*-Dm-Ac and *m*-Dm-Ac probes

Maleimide groups are known to react fairly selectively with thiols in addition reactions involving their C=C bond.[24,25] However, if a fluorogen bearing two maleimide groups that are separated by a precise distance were prepared, its latent fluorescence would only be excited when these two maleimide groups react with two thiol groups at the same time.[17] Therefore, on the basis of this mechanism, we designed two fluorescent probes for VSPPs, *o*-Dm-Ac and *m*-Dm-Ac, by employing an acridine dye as the fluorophore and two maleimide groups as a pair of reactive groups separated in accordance with the spatial separation of two active cysteine residues contained in the conserved amino acid sequence (–CGPC–) of human thioredoxin. As shown in Scheme 1, the two maleimide groups are located in the *ortho* positions of *o*-Dm-Ac and in the *meta* positions of *m*-Dm-Ac. The two probes were easily synthesized and characterized by elemental analysis, ^{1}H NMR and IR spectroscopy, and MS.

Spectroscopic properties and responses of probes to vicinal-sulfydryl-containing peptides

First, on the basis of the conserved amino acid sequence (–CGPC–) in human thioredoxin, we designed a series of small peptides as model molecules, including WCGPCK (W-6), WCGGPCK (W-7), and WCGGGPCK (W-8), which contain two cysteine residues separated by different distances (see Supporting Information). Then, the spectroscopic properties and responses of probes to small dithiol peptides were tested. As shown in Figure 1, the two probes themselves exhibit almost negligible background fluorescence. On introduction of small peptides into solutions of the probes, dramatic enhancements of the fluorescence intensity at 460 nm were observed on excitation at 360 nm (Supporting Information, Figure S1). The fluorescence change of

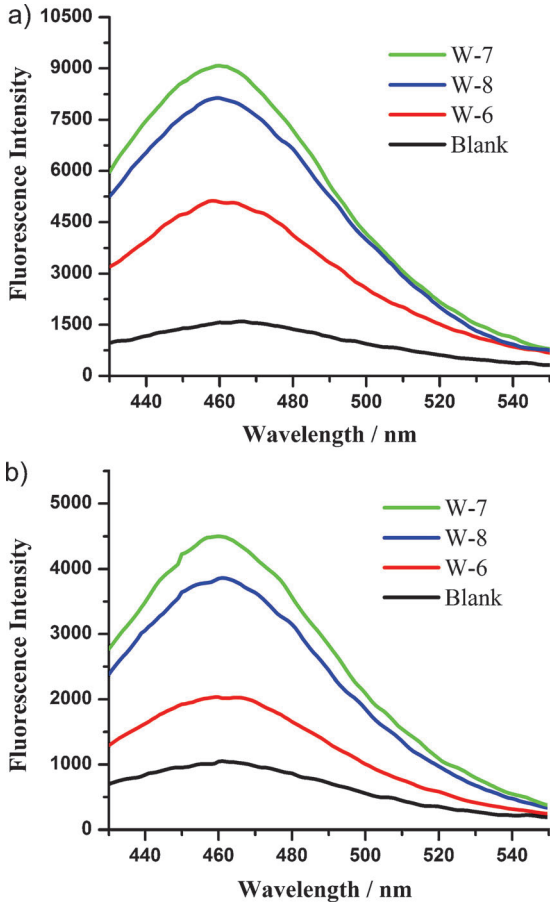

Figure 1. Fluorescence responses of 7 μM *o*-Dm-Ac (a) and 7 μM *m*-Dm-Ac (b) to 2 μM solutions of W-6, W-7, and W-8 in 20 mM PBS (pH 7.4). λ_{ex} = 360 nm, λ_{em} = 460 nm.

the reaction of *o*-Dm-Ac with small peptides (Figure 1a) is more pronounced than that of *m*-Dm-Ac (Figure 1b), and this can be attributed to the shorter distance between the two maleimide groups in *o*-Dm-Ac, which is a better match with the model peptides. We also found that the response of *o*-Dm-Ac to W-7 was the most sensitive (Figure 1a), so a detection method for W-7 as a typical vicinal-sulfydryl-containing peptide is discussed in detail below.

The influence of pH on the above reaction system was first examined (Supporting Information, Figure S2). This demonstrated that there is no significant variation in fluorescence intensity near physiological pH, that is, *o*-Dm-Ac can work well under physiological pH conditions. The fluorescence responses of *o*-Dm-Ac to W-7 at various concentrations under the optimized conditions (20 mM phosphate-buffered saline (PBS) with pH 7.4 and 7 μM of *o*-Dm-Ac; see Supporting Information, Figures S3 and S4) are shown in Figure 2. The fluorescence intensity increases with increasing W-7 concentration, and good linearity is obtained in the concentration range of 0–5.5 μM (Figure 2b). The regression equation is $F = 2283.29 + 3390.83 \times [\text{W-7}]/\mu\text{M}$ with a linear coefficient of 0.9989. The limit of detection is 25 pM (relative standard deviation ($n = 11$): 2.8%). The fluorescence kinetic curve of *o*-Dm-Ac reacting with W-7 is depicted in Figure S5 of the Supporting Information.

Fluorescence responses to vicinal-sulfydryl-containing proteins

The above results showed that *o*-Dm-Ac could react with small peptides containing two sulfydryl groups with appropriate distance between them in vitro. In living cells, such closely spaced sulfydryl groups often exist in vicinal-sulfydryl-containing proteins, which play a significant role in maintaining the cellular redox microenvironment.[4,26,27] Hence, we chose bovine serum albumin (BSA), an approximately 66 kDa protein with seventeen disulfide bridges and one free Cys residue, as a control protein to test the selective response of the probe *o*-Dm-Ac in vitro. Reduced BSA (rBSA) was prepared by reaction of BSA with tris(2-carboxyethyl)phosphine (see Supporting Information).

As expected, after the addition of 7 μM *o*-Dm-Ac to 2 μM solutions of BSA (or denatured BSA, data not shown), only a slight change in fluorescence was observed, which can be attributed to the effect of BSA itself on the solution environment. With 2 μM rBSA instead of BSA, a remarkable increase in fluorescence was observed (Figure 3a). Thus, the rBSA molecule must have some neighboring pairs of sulfhydryl

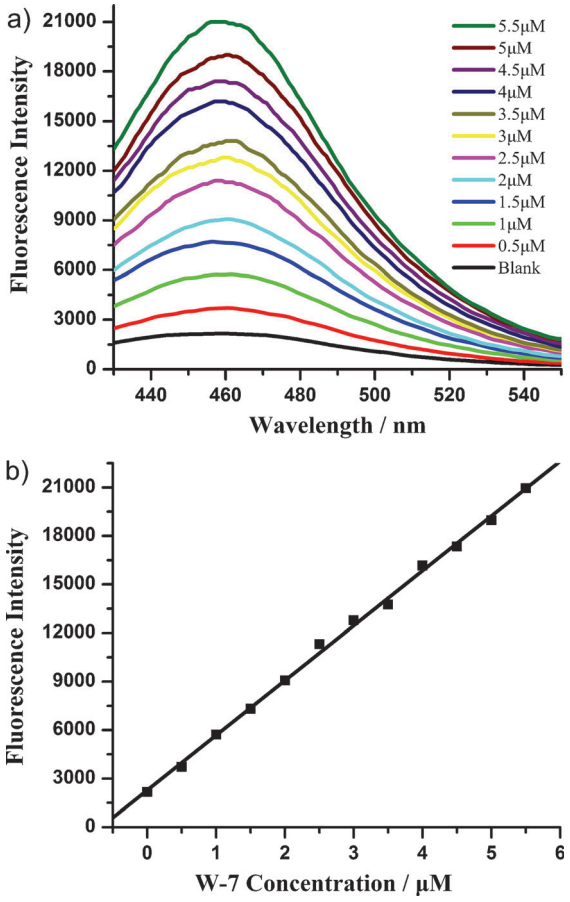

Figure 2. Fluorescence responses of 7 μM *o*-Dm-Ac toward various concentrations of W-7 from 0 to 5.5 μM at 460 nm. Spectra were acquired in 20 mM PBS (pH 7.4). $\lambda_{ex} = 360$ nm, $\lambda_{em} = 460$ nm.

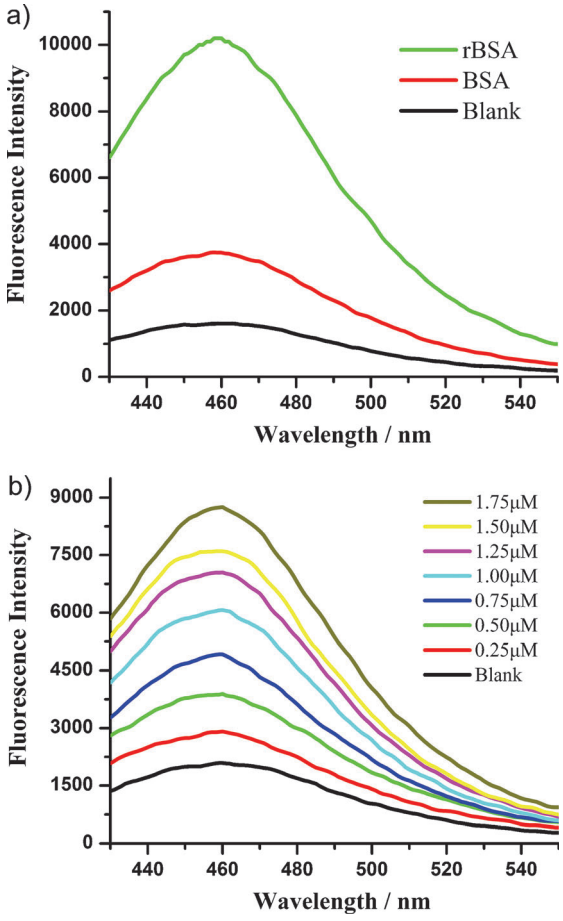

Figure 3. a) Fluorescence responses of 7 μM *o*-Dm-Ac to 2 μM solutions of BSA or rBSA. b) Fluorescence responses of 7 μM *o*-Dm-Ac toward to various concentrations of rBSA from 0 to 1.75 μM. Spectra were acquired in 20 mM PBS (pH 7.4). $\lambda_{ex} = 360$ nm, $\lambda_{em} = 460$ nm.

groups that match the two maleimide groups of our probe in position.

Next, the fluorescence response of *o*-Dm-Ac to rBSA at various concentrations was studied. The fluorescence intensity of *o*-Dm-Ac increased with increasing concentration of rBSA (Figure 3b), and the fluorescence intensity and rBSA concentration showed good linearity in the range 0–1.75 μM (Supporting Information, Figure S6). The regression equation is $F = 2036.17 + 3854.52 \times [\text{rBSA}]/\mu\text{M}$ with a linear coefficient of 0.9985. The limit of detection is 16 pM (relative standard deviation ($n = 11$): 2.1 %).

The fluorescence kinetic curve of *o*-Dm-Ac reacting with rBSA (Figure 4) shows that, after the addition of rBSA and *o*-Dm-Ac, fluorescence intensity increases quickly within 1 min and then reaches a plateau. This demonstrates that the probe can respond rapidly to rBSA, which is very important in monitoring concentration changes of VAPPs in situ.

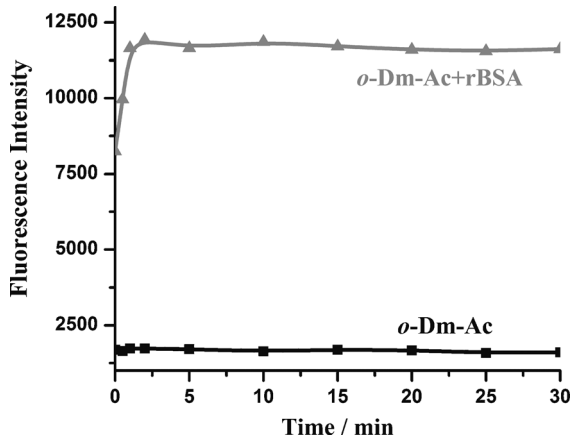

Figure 4. Kinetics study on the reaction of 7 μM *o*-Dm-Ac with 7 μM rBSA.

To confirm the specific labeling of vicinal dithiol in proteins by *o*-Dm-Ac, BSA and rBSA were treated with *o*-Dm-Ac, and then the reaction products were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). As shown in Figure 5, a remarkable fluorescence signal was observed in the lane loaded with rBSA, whereas no fluorescence was observed with BSA, which has only one free Cys residue (monoCys protein). This indicates that a monoCys protein can not restore the fluorescence of the acridine dye. When one maleimide group of the probe reacts with a monoCys protein, the other maleimide residue cannot combine with another protein molecule, due to the large size of the protein molecule. This experiment proves that *o*-Dm-Ac can only label selectively proteins with two sulfhydryl groups separated by the appropriate distance. To further evaluate the specificity of *o*-Dm-Ac for vicinal-sulfydryl-containing proteins, the reduced form of Trx (rTrx) was employed. It is a typical vicinal-sulfydryl-containing protein containing only one pair of vicinal sulfydryl groups. As expected, only one fluorescent band was observed in the lane loaded with rTrx and *o*-Dm-Ac, whereas the lanes loaded with oxidized thioredoxin (oTrx) exhibited a negligible

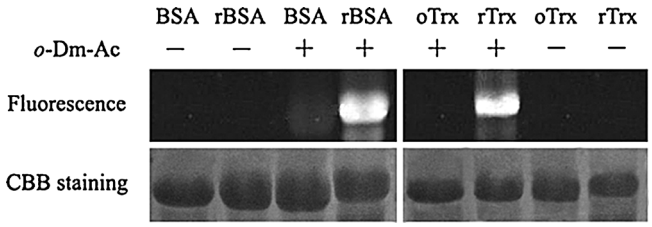

Figure 5. SDS-PAGE analysis of the specific labeling of vicinal dithiol groups in proteins by *o*-Dm-Ac. rBSA = reduced BSA (with vicinal dithiol groups), rTrx = reduced rTrx (with vicinal dithiol groups), oTrx = oxidized Trx (without thiol groups). +: the compound was present in the detection system; −: the compound was not present in the detection system.

fluorescence signal (Figure 5). As a control, these protein bands in the gel were stained with Coomassie brilliant blue. This further confirmed the specificity of *o*-Dm-Ac toward vicinal dithiols. In addition, the fluorescence response of *m*-Dm-Ac to rTrx was also studied under simulated physiological conditions. Compared with *o*-Dm-Ac, the *m*-Dm-Ac probe has a relatively low sensitivity to rTrx, albeit with a similar selectivity (data not shown).

Fluorescence imaging of VSPPs in living cells

o-Dm-Ac makes in situ imaging of endogenous VSPPs possible. HepG2 cells were incubated with *o*-Dm-Ac for 1 min and then washed three times with PBS to remove free probe before the fluorescence signal was collected. As shown in Figure 6, only a background signal was observed in the control (Figure 6a), whereas a strong fluorescence signal was detected in cells treated with *o*-Dm-Ac (Figure 6c). This indicates that *o*-Dm-Ac can penetrate cell membranes quickly and image intracellular dithiols. Considering that glutathione (GSH), one of the most abundant low molecular weight free thiols in cells,[28] might affect the imaging of proteins, an experiment to eliminate GSH was performed. As shown in Figure 6 e–h, after treatment with 1 mM l-buthionine sulfoximine (BSO) for 24 h at 37 °C,[29] cells were incubated with *o*-Dm-Ac, and a strong fluorescence signal was still observed (Figure 6g). To prove that BSO can completely remove GSH from cells, the concentration of GSH was measured (Supporting Information, Figure S7). This showed that GSH can be entirely removed from cells by treatment with 1 mM BSO for 24 h. These results demonstrate that *o*-Dm-Ac can be used to image endogenous VSPPs directly with rapid response in living cells.

To evaluate the potential toxicity of *o*-Dm-Ac to cells, an MTT assay was performed (Supporting Information, Figure S8). The cell viability did not significantly change on treatment, even with 20 μM of the probe at 37 °C for 24 h, and the half-maximal inhibitory concentration (IC_{50}) was 458 μM. The data demonstrate that no obvious toxicity is exerted by *o*-Dm-Ac on cultured cell lines under experimental conditions.

Conclusion

Active-site-matched probes were designed by regulating the positions of two active sites in a fluorescent molecule to

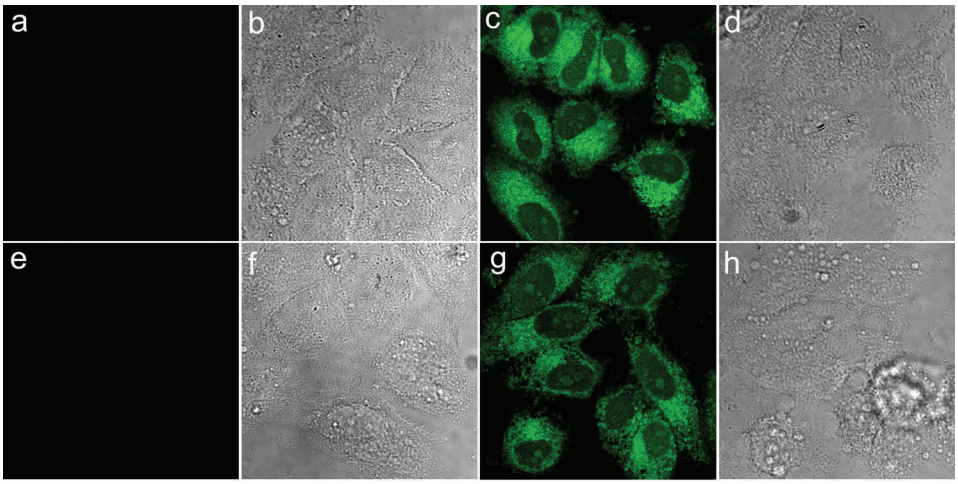

Figure 6. Confocal fluorescence images of HepG2 cells. a) Cells without *o*-Dm-Ac as a control. c) Cells incubated with 2 μM *o*-Dm-Ac for 1 min. e) Cells treated with 1 mM BSO for 24 h without *o*-Dm-Ac at 37 °C. g) Cells pretreated with 1 mM BSO for 24 h at 37 °C and then incubated with 2 μM *o*-Dm-Ac for 1 min. b), d), f), and h) Corresponding bright-field images.

match the distance between sulfydryl groups in a protein molecule. Thus, two active-site-matched fluorescent probes, *o*-Dm-Ac and *m*-Dm-Ac, were synthesized by regulating the spatial separation of two maleimide groups in a fluorescent dye to match that of two active cysteine residues in the conserved amino acid sequence (–CGPC–) of human thioredoxin. Experimental results showed that the two probes can specifically detect dicysteine-containing motifs in peptides and proteins, and *o*-Dm-Ac exhibits a higher sensitivity to W-7 and rTrx, which implies a positive correlation between active-site matching and sensitivity of detection. More importantly, *o*-Dm-Ac was successfully used to image VSPPs rapidly and directly both in vitro and in living cells. The probe-design strategy is instructive for the design of other protein probes, and our probe will provide a good chemical tool to help explore the potential function of VSPPs in living cells.

Experimental Section

Materials and apparatus

Bovine serum albumin (BSA), thioredoxin (Trx), L-buthionine sulfoximine (BSO), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), and tris(2-carboxyethyl)phosphine (TCEP) were purchased from Sigma-Aldrich. GSH-Glo Glutathione Assay was obtained from Promega. All other chemicals and solvents used were local products of analytical grade. Small peptides WCGPCK, WCGGPCK, and WCGGGPCK were provided by GL Biochem Ltd (Shanghai, P. R. China). Stock solutions (1.00 mM) of *o*-Dm-Ac and *m*-Dm-Ac were prepared by dissolution in DMSO. Ultrapure water (18.2 MΩ cm) was used throughout the analytical experiments. HepG2 cells were purchased from the Committee on Type Culture Collection of the Chinese Academy of Sciences. Elemental analysis was performed on an Element Analyzer (CHNS/O II, PerkinElmer, USA). ^{1}H NMR spectra were recorded on an AVANCE 300 spectrometer (Bruker, Switzerland). ESI mass spectra were recorded on a QTOF Micro YA 263 mass spectrometer. IR spectra were recorded on a Nicolet Nexus 770 spectrometer. Fluorescence spectra were obtained with an FLS-920 Fluorescence Spectrometer (Edinburgh Instruments, UK). Absorption spectra were recorded on a Varian Cary 100 UV/Vis spectrophotometer. The fluorescence images of living cells were obtained with a confocal laser scanning microscope (TCS SP5, Leica, Wetzlar, Germany). The MTT analysis was recorded on a microplate reader (Bio-Tek ELx800, USA).

Synthesis

Synthesis of *o*-Dm-Ac: First, compound **I** was synthesized according to our previous report.[30] In brief, *o*-phenylenediamine (0.7 g, 6.5 mmol), acridine hydrochloride (0.7 g, 3.2 mmol), and sulfur (0.45 g) were placed in a flask, and then the mixture of solids was slowly heated to 140 °C with vigorous stirring for about 4 h until no more H_2S gas was emitted. Then the melt was cooled and washed with diethyl ether (2× 50 mL). The black solid was extracted with 10% HCl (100 mL) to give a dark brown solution. The solution was basified with NH_3 (aq) and a brown solid was precipitated. The solid was collected by filtration and dried under vacuum at 50 °C to give **I** as a yellow-brown powder. The vicinal maleimide compound was prepared according to a previously published synthetic route.[17] Diamine **I** (373 mg, 1.31 mmol) and maleic anhydride (385 mg, 3.93 mmol) were placed in a dry 100 mL round-bottom flask. Chloroform (12 mL) was added and the solution was heated to reflux for 20 h. The mixture was then filtered to give **II** as a yellow powder, which was rinsed liberally with acetone and dried under vacuum. Acetic anhydride (10 mL) and sodium acetate (43 mg, 0.524 mmol) were added to this solid and the reaction was sustained for 2 h at 100 °C. The mixture was then cooled to 4 °C, stirred vigorously for 2 h, and filtered. The brown solid obtained was dried under vacuum and purified by recrystallization from DMF/H_2O to give *o*-Dm-Ac (0.5 g, 85%). IR (KBr): $\tilde{\nu}$ = 3100, 1717 cm^{-1}; ^{1}H NMR (300 MHz, DMSO): δ = 7.18–7.24 (d, 2H, J = 18 Hz), 7.59–7.65 (m, 5H), 7.71–7.75 (d, 4H, J = 12 Hz), 7.86–7.91 (d, 2H, J = 15 Hz), 8.23–8.26 ppm (d, 2H, J = 9 Hz); HRMS (ESI-TOF): m/z $[M+H]^+$ calcd: 446.4266; found: 446.4257; elemental analysis (%) calcd for $C_{27}H_{15}N_3O_4$: C 72.80, H 3.39, N 9.43; found: C 72.82, H 3.40, N 9.47%.

Synthesis of *m*-Dm-Ac: The protocol used to obtain *m*-Dm-Ac was followed, except that *m*-phenylenediamine was used instead of *o*-phenylenediamine. IR (KBr): $\tilde{\nu}$ = 3100, 1713 cm^{-1}; ^{1}H NMR (300 MHz, DMSO): δ = 6.74 (s, 1H), 7.18–7.30 (m, 4H), 7.48–7.59 (m, 4H), 7.70–7.81 (m, 4H), 8.13–8.16 ppm (d, 2H, J = 9 Hz); HRMS (ESI-TOF): m/z $[M+H]^+$ calcd: 446.4257; found 446.4251; elemental analysis (%) calcd for $C_{27}H_{15}N_3O_4$: C 72.80, H 3.39, N 9.43; found: C 72.79, H 3.42, N 9.47.

SDS-PAGE and fluorescence imaging of gels

The selectivity of the two probes was confirmed by 12% SDS-PAGE. Samples were labeled in PBS at 37 °C for 5 min with a probe-to-protein ratio of 4:1. After labeling, 20 μg of the protein per well was loaded in SDS-PAGE gel. Then, electrophoresis was

performed immediately, and the gel was imaged by a Herosbio Gel-Imaging System (excitation: 360 nm, emission: 460 nm). As a control, the same gel was stained by Coomassie brilliant blue after the fluorescent image was obtained.

Cell culture and cell labeling

HepG2 cells were cultured with RPMI-1640 medium (Gibco, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS) at 37 °C under a humidified atmosphere of 95% air and 5% CO_2. Cells in the logarithmic phase of growth were seeded on glass-bottom culture dishes the day before imaging to obtain a cell density of about 80%. For fluorescence imaging, the cells were labeled with 2 μM of *o*-Dm-Ac in FBS-free RPMI-1640 for 1 min and then washed three times with the PBS to remove unbound probe. Then the cells were imaged immediately with a confocal microscope.

Confocal imaging

Fluorescence imaging was performed with a TCS SP5 confocal laser scanning microscope (Germany Leica Co., Ltd) with an objective lens (×40). Excitation of probe-loaded cells at 405 nm was carried out with an argon laser, and emission light was collected with an META detector between 400 and 500 nm.

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Keywords: fluorescent probes · imaging agents · peptides · proteins · thiols

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