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Redox Proteomics

Highly Selective Fluorescent Probe for Vicinal-Dithiol-Containing Proteins and In Situ Imaging in Living Cells**

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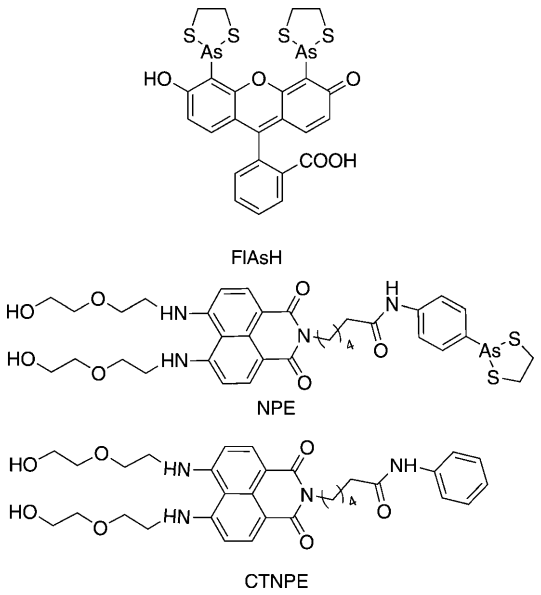
The protein thiol group is vulnerable to oxidation by reactive oxygen species and reactive nitrogen species, and plays a principal role in maintaining an appropriate oxidation–reduction state of the protein,[1] regulating signal pathways,[2] and responding to various diseases,[3] such as cancer,[4] diabetes,[5] and neurodegeneration.[6] As the reductive end of this redox buffer network, vicinal-dithiol-containing proteins (VDPs) are attracting more and more attention. VDPs are proteins that contain two thiol groups that are close to each other in space. In fact, most contain the thiol groups as a -CX_nC- (n: normally 2–6, X: amino acid) motif instead of a CC sequence.[7] In contrast to the situation when a single thiol group is present, the proximity of a second thiol group could promote immediate oxidation to form a disulfide following the transient intermediacy of an oxidative species, such as a sulfenic acid, a thiyl radical, or an *S*-nitrosothiol.[8] Therefore, as one final end of the redox buffer network, protein vicinal dithiols show a higher tendency to cope with redox changes and are more sensitive and prone to free-radical oxidation.[9]

Because of the function of protein vicinal dithiols, it is of considerable significance to measure the proteome of vicinal dithiols directly in biological systems. Since the first report of the selective binding of arsenic(III) with VDPs,[10] several strategies for detection and functional studies have been employed. Arsenite-affinity chromatography[11] could enrich VDPs and made proteomic detection possible. However, the highly reactive vicinal dithiols were exposed to an open and in vitro oxidative environment during the lysis and affinity purification procedures, and many underwent oxidation and disulfide exchange, which made data interpretation difficult. Biotinylated conjugates of phenylarsine oxide (PAO)[12] bound to VDPs immediately, but the application of these nonfluorescent conjugates with poor membrane permeability in the detection of VDPs in living cells without cell lysis was limited. VDPs were also traced by another redox proteomic methodology, whereby PAO and a thiol-alkylation agent were used to block vicinal dithiols and thiols. The vicinal dithiols were then labeled after the removal of PAO.[13] This method can be used to selectively detect VDPs in vitro through indirect procedures, but it is restricted by the stability of PAO itself, and cannot trace the VDPs directly in cells. Dimaleimide fluorogens were reported to react with a target peptide containing a vicinal dithiol and have been applied in protein labeling.[14] Biarsenical fluorescent analogues, such as FlAsH (Scheme 1), have been successfully employed in the imaging of proteins in living cells on the basis of the interaction of the arsenic centers with two pairs of vicinal thiols in tetracysteine motifs (CCXXCC) that were genetically fused to the target protein.[15] Both of these approaches focused on the development of strategies for the labeling of target proteins, and the target peptides containing vicinal dithiols were exogenously introduced onto the target proteins in cells. As endogenous vicinal thiols play a key role in redox homeostasis, there is an urgent need to develop a methodology for the selective and direct detection of VDPs in living cells.



Scheme 1. Chemical structures of FlAsH, NPE, and CTNPE.

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In this study, we selected naphthalimide as a fluorophore and cyclic dithiaarsanes as the binding group to design and synthesize a highly selective and cell-permeable fluorescent probe (NPE, Scheme 1) for the rapid detection and visualization of VDPs both in vitro and in living cells. In comparison with the presently available method of arsenite-affinity chromatography[11] for the detection of VDPs, the fluorescence of NPE ensures the more rapid and effective detection of VDPs through direct fluorescence readout without the need for cell lysis or purification steps. This approach enables protein vicinal dithiols to remain in their native state in both in vitro and living-cell assays. Furthermore, the noninvasive determination of VDPs by the use of NPE in living cells enables us to collect more information on the global localization, distribution, and dynamic changes of endogenous VDPs.

Our investigations began with the design of NPE for the selective detection of VDPs (Figure 1). Small-molecular fluorescent probes are widely used in detecting, tracing, and visualizing the function of proteins because of their insignificant steric bulk and fast labeling kinetics.[17] For the investigation of specific VDPs both in vitro and in living cells, the probe must be selective, stable, water-soluble, and cell-permeable.[18] PAO is a popular specific ligand for protein vicinal dithiols, but it is readily oxidized,[19] which results in the loss of specific affinity for vicinal dithiols. Furthermore, the high polarity of the $As(OH)_2$ group of PAO makes it cell-impermeable. In this study we used a more stable cyclic dithiaarsane as the ligand (red in Figure 1) for selective binding to VDPs and also to improve the lipophilicity of NPE. The crystal structure of the tolylarsenic 2,3-dimercaptopropanolate complex reveals that two sulfur atoms form a stable five-membered ring with the arsenic atom.[20] As monothiols in proteins have much lower affinity than vicinal dithiols for trivalent arsenic centers,[21] the interchange of 1,2-ethanedithiol (EDT) in cyclic dithiaarsanes of NPE with vicinal dithiols in proteins could selectively discriminate vicinal dithiols from other forms of thiols in proteins. Naphthalimide (Figure 1) was introduced as a fluorophore with a suitable excitation wavelength, stable fluorescence signal, and moderate quantum yield (Φ_F) under physiological conditions (see Table S1 in the Supporting Information). To tune the ratio of lipophilicity to hydrophilicity, we introduced a biocompatible diglycol amine (Figure 1), which enhances both the water solubility and the cell compatibility of the probe. A six-carbon-atom spacer (Figure 1) was used between the cyclic dithiaarsane and the naphthalimide fluorophore. NPE was synthesized in six steps; a control compound without a cyclic dithiaarsane (CTNPE, Scheme 1) was also synthesized (see the Supporting Information).

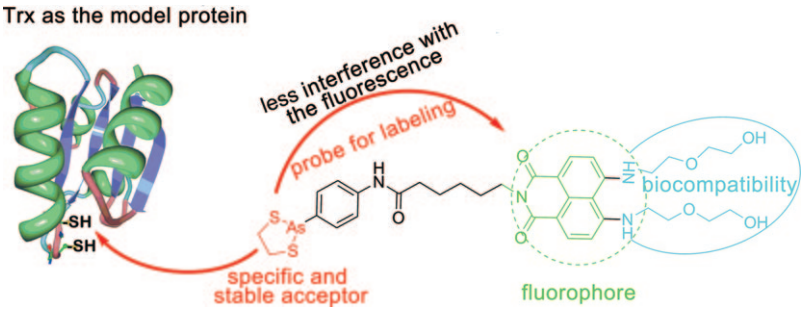

Figure 1. Design of NPE for the specific labeling of VDPs. Thioredoxin structure model: reduced human thioredoxin 2, chain A (PDB ID: 1W89).[16]

We next investigated the spectroscopic characteristics of NPE. The UV absorption and fluorescence intensity of NPE showed negligible change at pH 6–8 (see Figure S1 a,b in the Supporting Information). The UV absorption and fluorescence spectra of CTNPE were almost the same as those of NPE in HEPES buffer (see Figure S1c; HEPES = 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid); the Φ_F value of NPE was lower than that of CTNPE (see Table S1 in the Supporting Information). Thus, all in vitro tests were conducted in HEPES buffer (10 mM HEPES, 5 mM ethylenediaminetetraacetic acid, pH 7.4, 0.5% dimethyl sulfoxide). As VDPs are related to the redox environment of living cells, we next investigated the effect of redox agents on the fluorescence intensity of NPE. Different concentrations of dithiothreitol (DTT) and H_2O_2 induced little change in the fluorescence intensity of NPE (see Figure S1d). Thus, NPE exhibits stable fluorescence characteristics in various redox environments. Further experiments also demonstrated that NPE was stable and biocompatible (see Figures S2 and S5).

We then deduced a plausible mechanism for the selective detection of VDPs with NPE (Figure 2a). The exchange of EDT in the cyclic dithiaarsane of NPE with vicinal dithiols in VDPs induces covalent-bond formation between NPE and VDPs. To confirm this mechanism, we used NPE for the specific labeling of thioredoxin (Trx; see the amino acid sequence of Trx and its mutation in the Supporting Information). The reduced form of Trx (rTrx) is a typical VDP which only contains one pair of vicinal thiols. Different forms of Trx were incubated with NPE and CTNPE, and then separated by electrophoresis. A fluorescent band was observed only in the lane loaded with rTrx and NPE, whereas oxidized Trx (oTrx-1 and oTrx-2) or the monothiol mutant of Trx (rTrx-M) and its oxidized form (oTrx-M) exhibited no fluorescence signal (Figure 2b). Coomassie Brilliant Blue (CBB) staining demonstrated that the fluorescent band corresponded to the formation of an rTrx–NPE complex (Figure 2b). There was no fluorescent band observed when CTNPE, which lacks of the cyclic dithiaarsane moiety, was used for labeling. These results indicated that the five-membered dithiarsolane ring in NPE was cleaved by rTrx through the exchange of EDT in the cyclic dithiaarsane for the vicinal dithiol in rTrx to form the fluorescent rTrx–NPE complex. The results also demonstrated the selectivity of NPE for protein vicinal dithiols.

The selectivity, reversibility, reaction kinetics, and sensitivity of NPE were then explored in a fluorescence polarization (FP) assay. The "mix and measure" FP assay is applied extensively in studying the interaction of proteins with small

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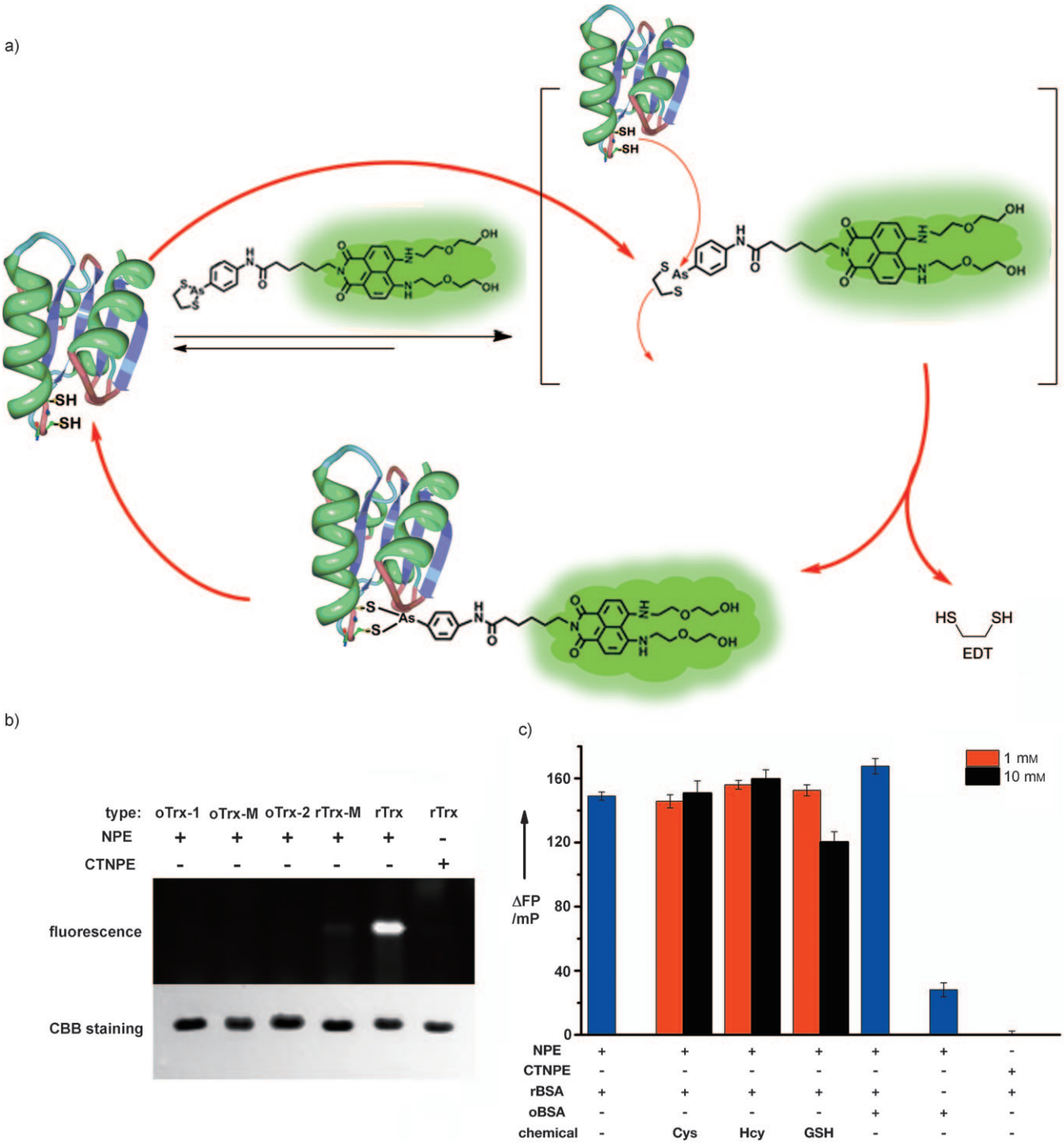

Figure 2. a) Plausible mechanism for the specific detection of VDPs with NPE. b) Selectivity of NPE as shown by SDS-PAGE. c) Selectivity of NPE in the FP assay. Blue bars: without the addition of Cys, Hcy, or GSH; red bars: with Cys, Hcy, or GSH (1 mM); black bars: with Cys, Hcy, or GSH (10 mM). FP data were acquired at 25 °C in HEPES buffer with an excitation filter (485 nm, 20 nm bandwidth) and an emission filter (528 nm, 20 nm bandwidth). The data shown are the average of quadruplicate measurements. Error bars: ± standard error of the mean (SEM); "+": the molecule was present in the detection system; "−": the molecule was not present in the detection system. rBSA = reduced BSA (with vicinal dithiols), oBSA = oxidized BSA (without thiols), oTrx-1 = thioredoxin (Trx) oxidized during expression and purification, oTrx-M = thioredoxin mutation (Trx C69G) oxidized immediately by hydrogen peroxide (H_2O_2), oTrx-2 = thioredoxin (Trx) oxidized immediately by hydrogen peroxide (H_2O_2), rTrx-M: reduced thioredoxin mutation (Trx C69G), rTrx = reduced thioredoxin.

molecules without the separation of free and bound ligands.[22] FP reflects the particle spinning and overall molecular weight of the fluorophore in solution; therefore, it is a convenient way to monitor the binding of NPE to VDPs (for a detailed discussion of the FP assay, see the Supporting Information). To enhance ΔFP (defined as the difference between the

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polarization value of free NPE or CTNPE and that of bound NPE or CTNPE) upon the binding of NPE to VDPs, we used a VDP with a medium molecular weight, bovine serum albumin (BSA), instead of thioredoxin as the model protein for a vicinal dithiol and disulfide.

On the basis of the FP signal of NPE, no labeling was observed with oxidized BSA (oBSA), in which the vicinal dithiol groups were oxidized (Figure 2c); however, a remarkable increase in the FP signal was observed after the addition of the reduced BSA (rBSA), whose disulfide groups were reduced to vicinal dithiols (Figure 2c). Negligible change in the FP signal was observed for CTNPE in the presence of rBSA (Figure 2c); thus, NPE could be used to selectively detect VDPs in the FP assay. Moreover, binding still occurred even in the presence of a high concentration of reduced glutathione (GSH), cysteine (Cys), or homocysteine (Hcy; Figure 2c). These results further confirmed the specificity of NPE toward vicinal dithiols.

The FP assay was used for further studies on the kinetics and reversibility of binding between NPE and VDPs. During the association of rBSA with NPE, the ΔFP value increased quickly within 2 min and then reached a plateau; this result demonstrates that the interaction of NPE with rBSA was nearly completed in 2 min (Figure 3a). Upon the addition of EDT to the complex, an approximately 80% decrease in the strength of the FP signal was observed within 10 min (Figure 3a). The ability to dissociate the rBSA–NPE complex was related to the concentration of EDT (Figure 3b). PAO, which competitively binds vicinal dithiols, decreased the FP response of NPE to rBSA in a dose-dependent manner (Figure 3c). A decrease in the ΔFP values was observed for the solution of NPE and rBSA in the presence of PAO within 10 min, which indicates that PAO inhibited the association of NPE with rBSA.

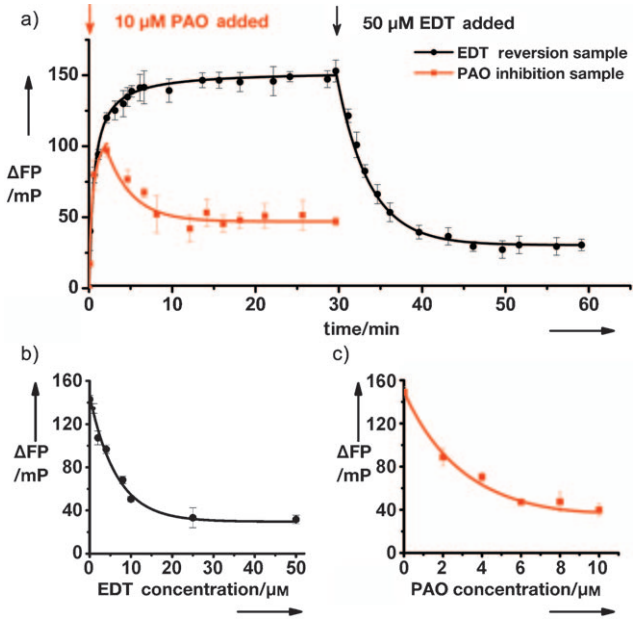

Figure 3. Association kinetics and reversibility of the association of NPE. a) Kinetics of binding of NPE to rBSA. EDT was injected into the detection system after NPE had been incubated with rBSA for 30 min, whereas PAO was coincubated with samples from the start. b) Effect of the dose-dependent dissociation of EDT on the association of NPE and rBSA. c) Dose-dependent inhibitory effect of PAO on the association of NPE and rBSA. ΔFP data were acquired at 25 °C in HEPES buffer with an excitation filter (485 nm, 20 nm bandwidth) and an emission filter (528 nm, 20 nm bandwidth). The data shown are the average of quadruplicate measurements. Error bars: ±SEM.

We next evaluated the sensitivity of NPE for rBSA. When NPE was treated with increasing amounts of rBSA, a substantial increase in the FP signal was observed. In contrast, for oBSA, negligible enhancement of the FP signal was observed. With CTNPE, the FP value was not changed upon the addition of increasing amounts of rBSA (see Figure S3a). We also observed that the detection limit of NPE for the rBSA is relatively low (1–150 nM), and that the FP signal increased linearly with the concentration of rBSA (see the inset in Figure S3b); therefore, the quantitative detection of rBSA over this concentration range may be possible. We determined the apparent dissociation constant (K_d) of the rBSA–NPE interaction to be $K_d = 0.14 \pm 0.02$ μM (see Figure S3b and details about the determination of the K_d value in the Supporting Information). Negligible change in the fluorescence intensity of NPE was observed during the in vitro assay (see Figures S1d and S4). Moreover, other common biological reductants, such as ascorbic acid and Cu^I, had negligible effect on the labeling of rBSA with NPE (see Figure S6). These results further demonstrated the substantial applicability of NPE for quantification of the changes in VDPs in living cells on the basis of changes in the fluorescence signal.

The FP assay was introduced for the selective detection of VDPs with NPE in vitro. This approach ensures that VDPs exist in their native state, as the FP assay requires no separation of the sample. The fast kinetics of binding between NPE and VDPs prevents the instability of vicinal dithiols in the process. The FP assay provides a rapid approach to the specific detection of VDPs in vitro, and the fast kinetics and reversibility of binding between NPE and VDPs also provide a suitable method for high-throughput screening for novel inhibitors of VDPs on the basis of this assay.

NPE makes in situ imaging possible for endogenous VDPs. By loading the cells with NPE, we ascertained the intracellular distribution of VDPs for the first time. A strong fluorescence signal was observed in cells treated with NPE (Figure 4a), but not for cells treated with CTNPE (Figure 4b), for which only a background signal was observed. These results indicated the specific binding of NPE to endogenous VDPs in living cells; this binding occurred through the reactive cyclic dithiaarsanes. To further explore this selective binding, we coincubated DTT or PAO with NPE in Chang liver cells. In agreement with the in vitro results (Figure 3a,c), the labeling of cellular vicinal dithiols with NPE was inhibited by PAO (Figure 4c) owing to the competitive binding of PAO with protein vicinal dithiols. Similarly, coincubation with DTT, which competitively binds to NPE, greatly decreased the labeling of cellular vicinal dithiols with NPE (Figure 4d). All these results demonstrated that cell-permeable NPE can be used for the in situ imaging of endogenous VDPs in living cells and to trace changes in

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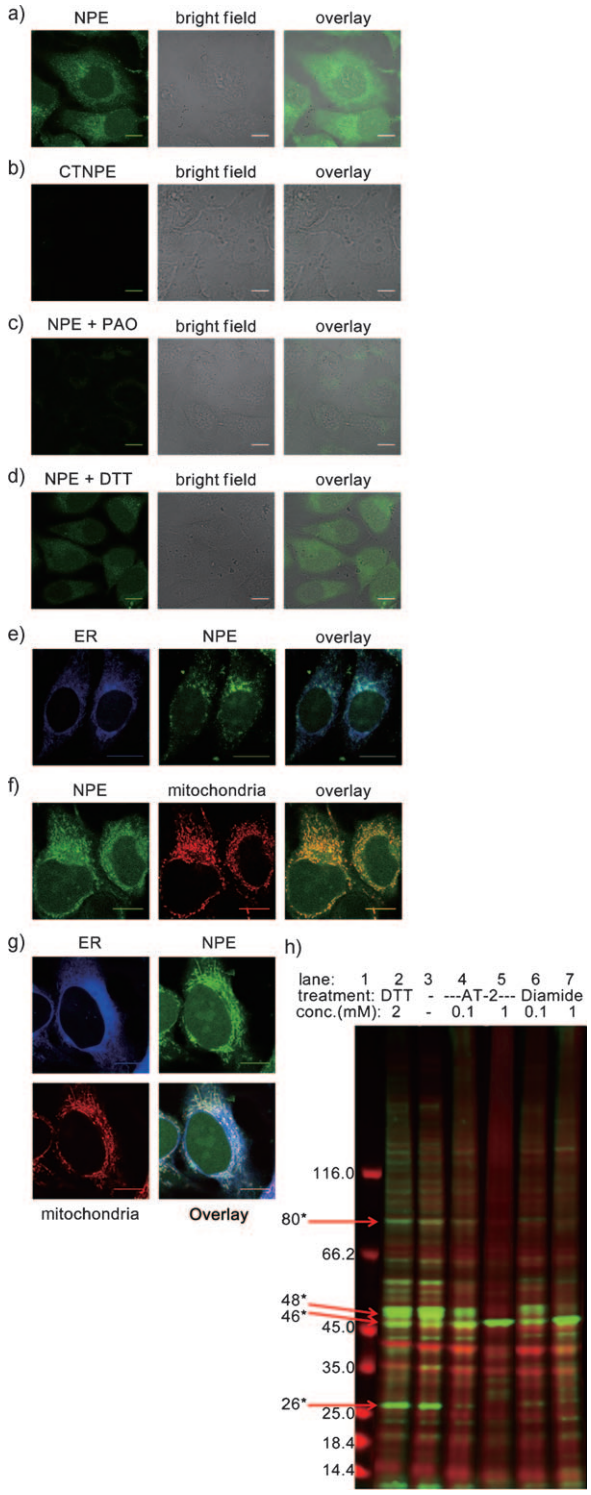

Figure 4. In situ imaging and cellular response of NPE-labeled Chang liver cells. a–d) Fluorescence of cells labeled with NPE (5 μM, a), CTNPE (5 μM, b), NPE (5 μM) in the presence of PAO (5 μM, c), and NPE (5 μM) in the presence of DTT (2 mM, d). e) Colocalization of NPE with ER-Tracker. f) Colocalization of NPE with Mito-Tracker Deep Red. g) Triple staining of the ER, mitochondria, and VDPs. h) Response of cellular vicinal dithiols in proteins to different redox environments induced by treatment with DTT (2 mM) for 12 h or treatment with Aldrithiol-2 (AT-2) or Diamide (0.1 or 1 mM) for 0.5 h. Green: NPE fluorescence signal; red: signal after staining with Sypro Ruby. Scale bar: 10 μm. *: molecular weight calculated by Quantity One software.

cellular VDPs on the basis of variations in fluorescence intensity.

The localization of intracellular VDPs was also investigated in Chang liver cells. When observed by confocal microscopy, the fluorescence signal of the NPE-labeled VDPs showed a punctuated pattern mainly concentrated in a small, eccentric perinuclear zone; the intensity was low in the cytoplasm and nucleus (Figure 4a). This pattern suggested mitochondrial localization, which was further confirmed by colocalization studies with subcellular organelle-specific labels, that is, ER-Tracker Blue-White DPX for the endoplasmic reticulum (ER) and Mito-Tracker Deep Red for mitochondria. The merged image of the fluorescence of NPE and mitochondria stained with Mito-Tracker Deep Red showed convincing yellow fluorescence in a punctuated pattern (Figure 4f), which implied the colocalization of the VDPs with mitochondria. Some colocalization of the VDPs with the ER was also observed (Figure 4e), but to a much lesser extent than with the mitochondria.

Triple staining also supported the view that the VDPs were mainly localized in the mitochondria (Figure 4g). The individual fluorescence signal for the ER (blue) was largely preserved, and the mitochondria (red) appeared yellow, which convincingly indicated colocalization with the NPE signal.

It is interesting that protein vicinal dithiols seem to be abundant in mitochondria, as demonstrated by NPE labeling of living cells. In a previously described image of a FlAsH-labeled exogenous tetracysteine tracer,[15a] a weak background signal also came from mitochondria, maybe as a result of weak binding of the probe with vicinal dithiols. The proteomic identification of arsenic-binding proteins in cell lysate also proved the existence of abundant mitochondria-related proteins.[12b] As the key organelle for respiration, mitochondria are the main site for many redox-related life processes. We previously reported that *S*-nitrosoproteins exist mainly in the mitochondria and perimitochondrial compartment, which suggests that mitochondrial proteins are prone to modification and functional regulation in the presence of nitric oxide (NO).[23] An image of the protein disulfide proteome in mammalian cells also showed a weak signal in mitochondria that markedly increased upon oxidative challenge.[24] Thus, these disulfides remained in the reductive form (vicinal dithiols) in their normal state. Furthermore, both *S*-nitrosothiol and disulfide formation are determined by mitochondrial respiration and the generation of reactive oxygen species. Now we have further shown the mitochondrial localization of cellular VDPs by in situ labeling and imaging. These results suggest that mitochondria are the central participants in thiol redox regulation and may have profound effects on protein function.

By using this live-cell-labeling technique, we further showed that the level of intracellular protein vicinal dithiols changed upon redox regulation. The fluorescence intensity of NPE-treated cells increased gradually from that of DTT-untreated cells as the concentration of DTT was increased. In contrast, the fluorescence intensity of NPE-treated cells decreased as the concentration of Diamide was increased (see Figure S7).

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Electrophoresis provided further information on the different responses of different proteins. The signal observed upon staining with Sypro Ruby (red) showed no clear difference in protein abundance between the samples, but significant variation was revealed by the signal of VDPs labeled with NPE (green). Furthermore, different proteins responded to redox changes with a different pattern. For example, after treatment with a reductant for 12 h, proteins with a molecular weight larger than 80 kDa showed increased reduction. The signals for vicinal dithiols on the 80 kDa, 48 kDa, 28 kDa, and other proteins decreased under oxidative conditions in a dose-dependent manner; however, that of the vicinal dithiol of the 46 kDa protein even increased slightly after oxidation (Figure 4h). These results suggest that the NPE labeling technique will be useful for the characterization of VDPs in living cells and the proteomic-scale identification of VDPs under physiological and pathological conditions.

In summary, we designed and synthesized a naphthalimide-based fluorescent probe, NPE. This probe acted as a tool for the rapid detection and imaging of VDPs both in vitro and in living cells. Direct determination of the status of protein thiol modification in situ is of considerable significance. Previously, it was possible to directly label protein sulfenic acids with newly synthesized fluorescent probes to determine protein sulfenation.[25] Herein, we have described an approach for the direct detection of protein vicinal dithiols with the fluorescent probe NPE. This approach permits the noninvasive study of VDPs both in vitro and in living cells. The results offer a method for the identification of VDPs in situ and insight into their potential roles in cell function. On the basis of this methodology, further studies on the redox regulation of signal pathways related to VDPs are currently under way, and a further proteomics study of VDPs with NPE is planned.

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