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A Ratiometric Fluorescent Probe for Imaging of the Activity of Methionine Sulfoxide Reductase A in Cells

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Abstract: *Methionine sulfoxide reductase A (MsrA) is an enzyme involved in redox balance and signaling, and its aberrant activity is implicated in a number of diseases (for example, Alzheimer's disease and cancer). Since there is no simple small molecule tool to monitor MsrA activity in real time in vivo, we aimed at developing one. We have designed a BODIPY-based probe called (S)-Sulfox-1, which is equipped with a reactive sulfoxide moiety. Upon reduction with a model MsrA (E. coli), it exhibits a bathochromic shift in the fluorescence maximum. This feature was utilized for the real-time ratiometric fluorescent imaging of MsrA activity in E. coli cells. Significantly, our probe is capable of capturing natural variations of the enzyme activity in vivo.*

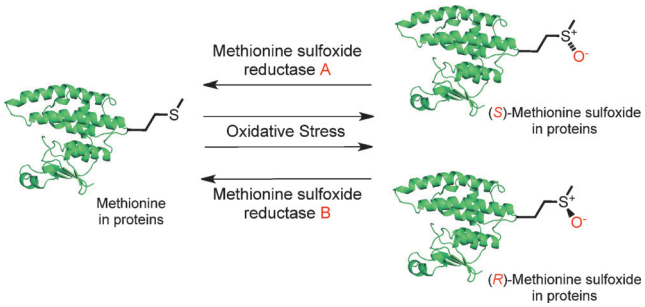

Figure 1. Representation of the reversible oxidation of methionine residues in proteins.

Methionine sulfoxide reductases (Msrs) are important enzymes, which are conserved throughout the tree of life and are responsible for the reduction of free and protein-bound methionine sulfoxide back to methionine.[1,2] As methionine sulfoxide has a chiral center at the sulfur atom, organismal oxidation of methionine to methionine sulfoxide caused by reactive oxygen species can lead to a mixture of (*R*)- and (*S*)-epimers. Nature has evolved two distinct classes of Msrs: MsrA, which reduces the (*S*)-epimer, and MsrB, which reduces the (*R*)-epimer (Figure 1).[3,4]

The importance of this class of enzymes has been demonstrated by knockout studies of MsrA in a number of organisms (bacteria, yeast, and mice), in which it has been associated with an increased susceptibility to oxidative stress.[5–8] On the other hand, overexpression of MsrA increases resistance to oxidative stress in cells, plants, and *Drosophila*.[9–12] Notably, overexpression of MsrA in *Drosophila* almost doubles its lifespan. Interestingly, the overexpression of MsrB in the *Drosophila* animal model had no effect on its aging.[13] It is clear that a large variety of cellular proteins are deactivated by methionine oxidation; however, an increasing number of examples have been described in which the generation of methionine sulfoxide is a gain-of-function posttranslational modification[14–16] At the heart of this important regulatory mechanism lie Msrs that have been shown to play a significant role in the development of neurological disorders (Alzheimer's disease and Parkinson's disease) and cancer.[17–19] It is therefore of great importance to have a simple and robust tool to monitor Msr activity in real time both in vitro and in vivo. Despite the utility of current probes that are based on radio or fluorescently labeled methionine sulfoxide,[20,21] they can only capture Msr activity in vitro. Recently, an elegant molecular biology tool for in vivo imaging of methionine sulfoxide has been described.[22] Complementary to this fluorescent protein-based sensor would be a small molecule fluorescent probe for Msr, which would provide direct information on Msr activity. As it is mainly MsrsA that were previously associated with health disorders and aging, we aimed at targeting the activity of this particular class of enzymes. Herein, we describe the development of ratiometric fluorescent reporter (*S*)-Sulfox-1 that enables monitoring MsrA activity in real time and capturing natural variations in the activity of this enzyme in vivo.

MsrA has been shown to reduce methyl *p*-tolyl sulfoxide in an enantioselective fashion.[23] The reduction of sulfoxide to sulfide provides a significant change of the electronic nature of the functional groups, which has also been shown to alter the spectral characteristics of various chromophores.[24–27] Thus, we have decided to attach the methyl phenyl sulfoxide moiety to a suitable fluorophore to construct the fluorescent reporter. We have opted for the BODIPY fluorophore because it is very bright, stable, environmentally insensitive, accessible, and modular.[28,29] Aryl substituents in the positions next to the nitrogens of the BODIPY core cause a bathochromic shift in fluorescence as the conjugated system is extended.[30,31] Moreover, functional groups on the aryl

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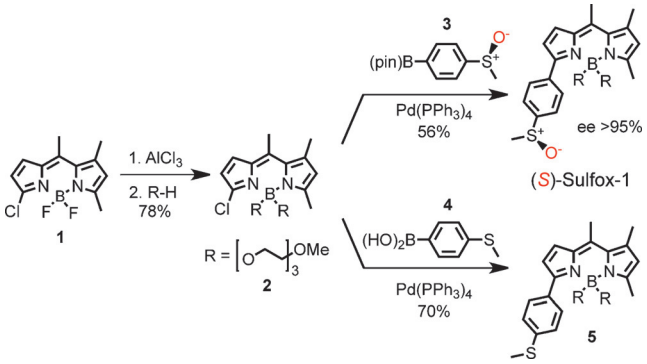
substituent can further affect the emission maxima, with electron donating groups increasing the red shift.[32] We hypothesized that reduction of the methyl phenyl sulfoxide moiety attached to the BODIPY core would red-shift the emission maximum and thus serve as a ratiometric probe for MsrA. The ratiometric nature of the probe would be ideal for in vivo imaging because the disadvantages of intensity probes, such as uneven cellular distribution and background fluorescence are inherently canceled out.

The (*S*)-Sulfox-1 probe was synthesized starting with 3-chloro-BODIPY **1**, which was prepared by an established procedure (Scheme 1).[32] The fluorine atoms were then



Scheme 1. Synthesis of (*S*)-Sulfox-1 and sulfide **5**.

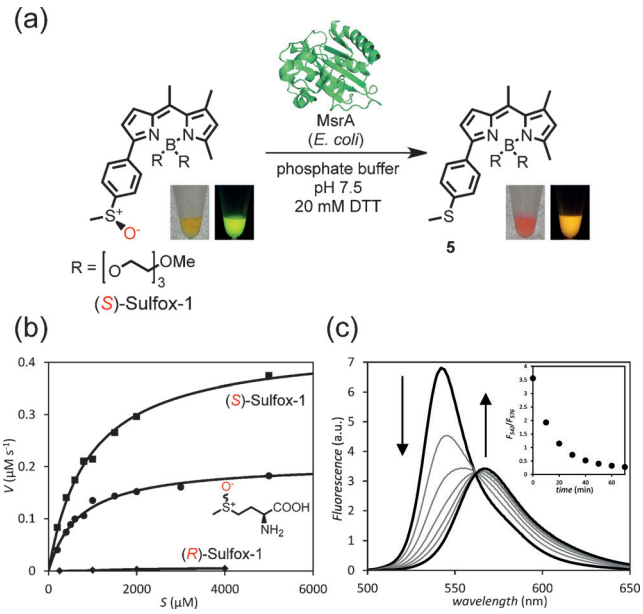


Figure 2. a) A sensing mechanism of (*S*)-Sulfox-1. b) Michaelis–Menten kinetics for MsrA with both (*S*)- (■) and (*R*)- (◆) enantiomers of Sulfox-1 and the natural substrate (*S*)-methionine-(*R*,*S*)-sulfoxide (●) measured in 50 mM sodium phopsphate buffer with 20 mM DTT, pH 7.5, at 37 °C. c) Changes in the emission profile of (*S*)-Sulfox-1 (40 μM) upon reduction with MsrA (2 μM) for 70 min at 20 °C (excitation at 510 nm). The inset shows changes in the emission ratio F_{542}/F_{576} as a function of time.

substituted with triethyleneglycol arms to increase the solubility and brightness in aqueous environments. The synthesis was completed by a Suzuki coupling with (*S*)-boronate **3**, which was prepared through an asymmetric oxidation/borylation sequence. The (*R*)- isomer of Sulfox-1 was prepared in the same way (see the Supporting Information), and sulfide **5** was obtained by a Suzuki coupling of 3-chloro-BODIPY **2** with commercial boronic acid **4**.

The spectroscopic characteristics of (*S*)-Sulfox-1 and sulfide **5** were assessed in phosphate buffer at pH 7.5. (*S*)-Sulfox-1 showed a maximum absorption at 509 nm ($\varepsilon = 3.6 \times 10^4\,\text{M}^{-1}\text{cm}^{-1}$) and a strong emission band at 541 nm with a quantum yield of 0.6. In contrast, the absorption maximum of sulfide **5** was at 527 nm ($\varepsilon = 2.9 \times 10^4\,\text{M}^{-1}\text{cm}^{-1}$) and the emission was red-shifted by 25 nm, which resulted in a maximum at a wavelength of 566 nm and a quantum yield of 0.5. The spectroscopic data gave us confidence that (*S*)-Sulfox-1 could be used as a ratiometric fluorescent probe. Next, recombinant *E. coli* MsrA was expressed and purified as a fusion protein with a GST tag, and its activity was tested against both the enantiomers of Sulfox-1 and the natural substrate methionine sulfoxide. (*S*)-Sulfox-1 proved to be a good substrate for MsrA and the proposed sensing mechanism was confirmed by HPLC-MS analysis (Figure 2a and the Supporting Information, Figure S8). Michaelis–Menten kinetics for (*S*)-Sulfox-1 gave $k_{cat} = 0.75\ \text{s}^{-1}$ and $K_M = 913$ μM, which are values similar to those for the natural substrate methionine sulfoxide ($k_{cat} = 0.37\ \text{s}^{-1}$ and $K_M = 700$ μM) (Figure 2b). On the other hand, under the assay conditions, no conversion was observed for the (*R*)-isomer of Sulfox-1 beyond a minor background reaction with DTT. The ability of (*S*)-Sulfox-1 to monitor MsrA activity in real time is demonstrated by the observed emission ratio at 542 nm and 576 nm (F_{542}/F_{576}). During the course of the reaction, that ratio was found to change from 3.8 to 0.3, a 13-fold difference (Figure 2c). Reports have indicated that certain sulfoxides can be racemized photochemically.[33,34] However, in our case, (*S*)-Sulfox-1 showed neither measurable racemization nor photodegradation during extended exposure (1 h) to 365 nm UV light (hand lamp, 8 W) in phosphate buffer in an NMR tube.

To show the utility of (*S*)-Sulfox-1 for in vivo imaging of MsrA activity, an *E. coli* strain with an artificially elevated level of the MsrA that was used for the recombinant preparation of the MsrA enzyme was utilized. The IPTG-induced cells were compared with the cells grown in the presence of glucose to maximize catabolite repression of the IPTG inducible promoter. After a 5 min staining of bacterial cells with (*S*)-Sulfox-1, the IPTG-induced cell population showed a substantial change in the fluorescence as compared with the uninduced cell population (Supporting Information, Figure S11). No change in the fluorescence ratio F_{535}/F_{570} was observed for (*R*)-Sulfox-1.

The next goal was to capture the natural variation of the MsrA activity in vivo. As previously reported, MsrA activity increases in *E. coli* cells with the growth phase, as measured in the cell lysate.[35] Thus, *E. coli* (BL21) cells were grown to $OD_{600} = 0.5$ and $OD_{600} = 3.5$, and these two populations were subjected to our staining protocol with (*S*)-Sulfox-1. After a 1-h incubation, the population of cells grown to $OD_{600} = 3.5$

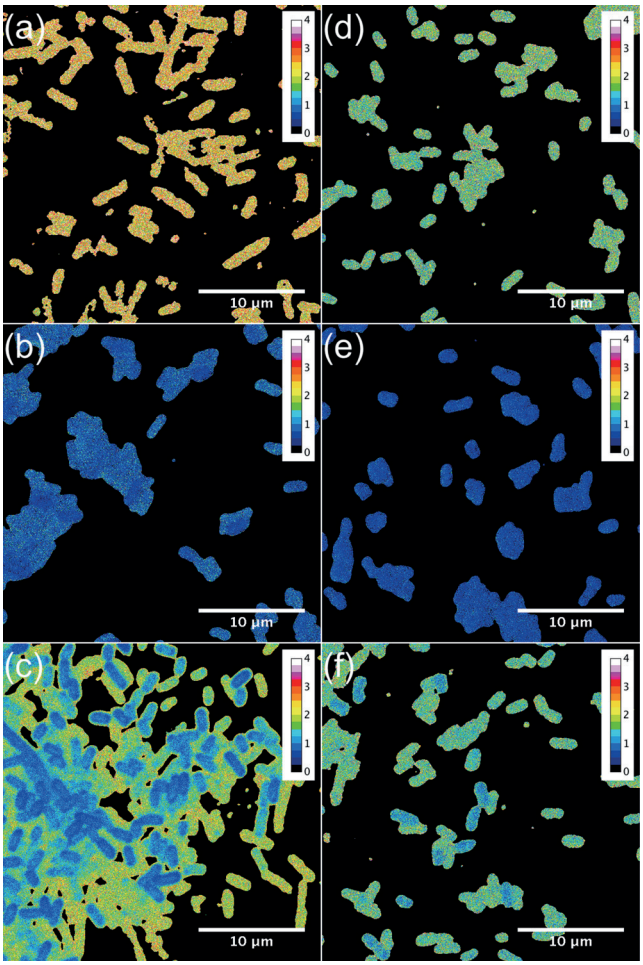


Figure 3. Pseudo-colored ratiometric confocal fluorescent images of *E. coli* cells stained with (*S*)-Sulfox-1 (100 μM in 1× TBE) for 1 h. a) $OD_{600}=0.5$ cell population. b) $OD_{600}=3.5$ cell population. c) A mixed sample of the $OD_{600}=0.5$ and $OD_{600}=3.5$ cell populations. d) $OD_{600}=3.5$ cell population with 5 mM (*S*)-**6**. e) $OD_{600}=3.5$ cell population with 5 mM (*R*)-**6**. f) A mixed sample of $OD_{600}=3.5$ cell population with 5 mM (*S*)-**6** and (*R*)-**6**. Excitation wavelength = 514 nm (laser). Emission was collected in two optical channels at 524–546 nm and 560–580 nm.

showed a significant decrease in the F_{535}/F_{570} ratio as compared with the $OD_{600}=0.5$ cell population (Figure 3a–c). Two populations of cells can be clearly distinguished in a mixed sample of $OD_{600}=0.5$ and $OD_{600}=3.5$ cells. To further verify the specificity of (*S*)-Sulfox-1 against MsrA, staining of the $OD_{600}=3.5$ cell population was performed in the presence of competitive non-fluorescent substrates (*S*)-**6** and (*R*)-**6** (see the Supporting Information). The presence of (*R*)-**6** did not have any effect on the decrease of the F_{535}/F_{570} ratio. On the other hand, (*S*)-**6** significantly inhibited the decrease of the F_{535}/F_{570} ratio, which further indicates the desired specificity (Figure 3d–f). These experiments establish that (*S*)-Sulfox-1 probe can indeed be utilized for capturing natural variation of MsrA activity in cells.

In summary, we have developed a fluorescent probe capable of real-time imaging of MsrA activity in cells. The fluorescent probe (*S*)-Sulfox-1 is bright, photostable, water soluble over a wide range of concentrations, and sensitive enough to capture natural variations in the activity of MsrA in cells. Since MsrA is implicated in a number of serious illnesses and is an important player in the redox balance and signaling, we believe our (*S*)-Sulfox-1 probe will provide a simple platform for monitoring MsrA activity in vivo and thus help uncover previously untraceable molecular biology relationships of these enzymes.

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