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Short communication

Mapping hydrogen sulfide in rats with a novel azo-based fluorescent probe

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ABSTRACT

We report herein a reaction-based fluorescent switch-on sulfide sensor, **azo3**, for the quantification of endogenous sulfides in rat tissues. The sensor was exploited based on the novel azo-sulfide chemistry and designed by locking the rhodol fluorophore into its nonfluorescent form with an azo group. However, the azo group would undergo a specific and biocompatible reaction with sulfides, triggering significant fluorescence increasements which were linear to the concentrations of sulfides. **Azo3** distinguished by its high sensitivity (148-fold fluorescent switch-on response), good selectivity (22-fold more selective towards sulfides than other bio-thiol species) and low detection limit (500 nM). Moreover, the **azo3**-based assay for biological sulfides displayed the unique advantage of being insusceptible to ultraviolet (UV) irradiation. **Azo3** has been successfully applied to the quantification of endogenous sulfides in rat plasma and tissues including heart, brain, liver, spleen, lung and kidney. In addition to providing **azo3** as a valuable tool to analyze sulfides in biological samples, we also discussed the influences of the electron effect on the sensitivity of the probes, which would shed some light on the design of future reaction-based probes.

1. Introduction

The last decade has witnessed the emerging and dramatic development of reaction-based small-molecule fluorescent probes for tracing cations, anions, amino acids, and small neutral molecules in complex biological specimens (Ueno and Nagano, 2011; Kobayashi et al., 2010; Li et al., 2014; Yang et al., 2013; Chan et al., 2012; Guo et al., 2014). These probes, judiciously designed by taking advantage of the specific probe–analyte reactions that invoke a change in the luminescent emission properties, usually offer the great advantages of high specificity, good sensitivity, and high temporal/spatial resolution. All these attributes combined with their nondestructive nature, have made reaction-based fluorescent probes versatile tools for monitoring, quantifying, and even imaging specific ions or molecules in living systems, thus have contributed considerably to interrogating the physiology and pathology of bio-analytes in their native environments (Nadler and Schultz, 2013).

Among the various biological species for which specific probes have been developed, hydrogen sulfide (H_2S) received the most research attention due to its newly updated identity as the third gasotransmitter after nitric oxide (NO) and carbon monoxide (CO) (Wang, 2003; Li and Moore, 2007; Li et al., 2011). Used to be notorious for its toxicity and the stink of rotten-eggs, the reputation of H_2S is enjoying a facelift, with increasing numbers of reports that it is endogenously produced in mammal tissues to exert fine, modulatory control over a range of biological processes (Hosoki et al., 1997; Zhao et al., 2001; Qu et al., 2008; Elsey et al., 2010). To determine local H_2S concentrations accurately in living cells, which is essential for detailed biological function study, a number of reaction-based fluorescent probes have been devised by making use of its nucleophilic reactivity to attack activated electrophiles (Qian et al., 2011; Liu et al., 2011, 2012), or its high affinity towards Cu^{2+} to remove the cation from quenched Cu–probe complexes (Sasakura et al., 2011; Hou et al., 2012; Qu et al., 2013), or its reductive property to reduce azides on masked fluorophores (Lippert et al., 2011; Peng et al., 2011; Yu et al., 2012; Montoya and Pluth, 2012; Chen et al., 2012; Das et al., 2012). Among these three principles, the redox reaction between H_2S and a fluorophore-tethered azide is most widely applied because azide-based H_2S probes generally show better selectivity than electrophilic probes and are more suitable to quantify H_2S than Cu–complexed probes. However, the azide group as a reaction trigger is challenged by its inherent photosensitivity. As a well-known photoaffinity label, an aryl azide is readily converted to a

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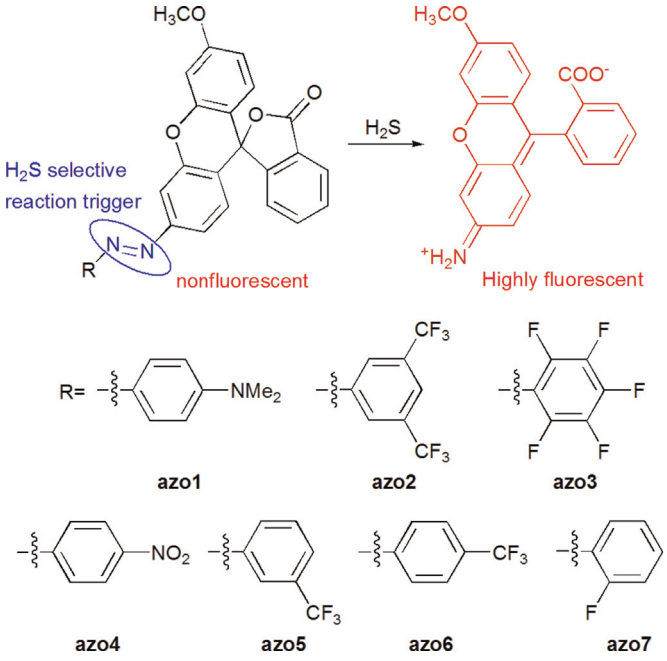

Fig. 1. Structures of the probes and the design philosophy.

nitrene upon photolysis (Fleet et al., 1969). Although this photosensitivity is generally non-problematic for routine detection, some reported probes do undergo photoactivation when exposed to continuous excitation, incurring a massive increase in the fluorescence and therefore complicating the detection (Bailey and Pluth, 2013). As a structurally similar cousin of the azide group, an azo group can be reduced to an amine under various reductive conditions. This inspired us to speculate its feasibility as an alternative to the azide group to act as a H_2S selective reaction trigger. For this purpose, seven azobenzene-caged rhodol analogues bearing different electron withdrawing groups, namely **azo1**–**7**, were designed and synthesized (Fig. 1). We envisioned that the azo group would lock the xanthene platform in a closed and non-fluorescent lactone form, while the reduction of the azo group by H_2S would release the aniline and therefore restore the probe's fluorescence, while the azo-fused phenyl groups with different degree of electron withdrawing effects may serve to tune the reactivity of the probes.

2. Material and methods

2.1. Materials and instruments

Information on the materials and instruments employed for the synthesis and elucidation of **azo1**–**7** can be found in the supplementary information part.

2.2. Synthesis of *azo1-7*

The probes were readily prepared *via* Mills reaction (Anspon, 1945) between substituted nitrosobenzenes and *O*-methyl rhodol. Procedures and spectra data were detailed in the supplementary information part.

2.3. Fluorometric analysis

All fluorescence measurements were carried out at 37 °C in phosphate buffer solution (PBS, 10 mM, pH 7.4, 50% ethanol) which was prepared with deionized water and purged with nitrogen for 5 min before use. The probes were dissolved in ethanol to make a 1.0 mM stock solution, which was diluted to the required concentrations for measurements. Sodium bisulfide (NaHS) dissolved in the above mentioned deoxygenated PBS was used as an aqueous sulfide source (stock solution: 10.0 mM). All fluorometric experiments were performed in triplicate.

2.4. Animal experiments

All animal procedures are approved by the Institutional Animal Care and Use Committee at Zhejiang University. Adult male Sprague-Dawley (SD) rats weighing 200–300 g were purchased from Laboratory Animal Center of Zhejiang Province and housed in the animal care facility at Zhejiang University before the experiment and provided with food and water ad lib. Rats were sacrificed and tissues were immediately removed. Tissue samples for detection were then prepared and analyzed as described in the figure caption part.

3. Results and discussion

3.1. Fluorescence properties of *azo1–7* before and after the treatment with NaHS

As expected, these probes, without exception, were barely fluorescent (20 μM) in PBS buffer (10 mM, pH 7.4, 37 °C). However, they responded differently to NaHS (500 μM). For **azo1**, the treatment of NaHS could not trigger any obvious fluorescence intensity (FI) increase, while **azo2** and **azo3** stood out by producing 103- and 148-fold turn-on responses respectively within 1 h of reaction in the emission maximum at 517 nm when excited at 468 nm (Fig. S1). For **azo4**–**7**, only slight fluorescence intensity increase was observed (Fig. S1). These results demonstrated the great impact of the electronic effect on the reactivity of the probes, and also implied the potential of **azo2** and **azo3** to monitor H_2S.

3.2. Reaction kinetics of *azo2* and *azo3*

The reaction kinetics of **azo2** and **azo3** were measured by recording the fluorescent spectra of the probe–NaHS system after different incubation time. As shown in Fig. 2a, the fluorescence intensity of the **azo2** system evenly increased as the time of the incubation extended and a linear relationship between the fluorescence intensity and the incubation time was observed within the first 3 h (Fig. 2b). We first hypothesized the reaction might be zero order overall. To check this hypothesis, a contrast experiment in which the initial concentration of **azo2** was decreased from 20 μM to 5 μM was performed. It turned out the latter went much more slowly (Fig. 2b). These results taken together illustrated that the reaction was by no means zero order and the linear relationship between the fluorescence enhancement and the reaction time was only due to the slow kinetic. When time-dependent fluorescence enhancement experiment was carried out for **azo3**, encouraging results were obtained which showed the system reached its maximum response in about 2 h, accompanied by a 225-fold increase factor (Fig. 2c and d). These results further confirmed that the electron deficiency of the azobenzene group was crucial to the reaction between the probes and sulfides, and that the more the electron deficiency was, the faster the reaction kinetic was.

3.3. Detection mechanism

To verify the detection mechanism, a liquid chromatography-high resolution mass spectrometry (LC-HRMS) analysis was applied to monitor the reaction. It turned out that both **azo2** and