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Two-photon fluorescent probe for hydrogen sulfide based on a red-emitting benzocoumarin dye

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ABSTRACT

Hydrogen sulfide (H_2S) is an endogenous gasotransmitter and plays intriguing biological roles. To study the biological role of H_2S, efficient fluorescent probes are in great demand. For imaging of H_2S in deep-tissue, a two-photon probe that emits in the red wavelength region is of choice to avoid the autofluorescence from intrinsic biomolecules. Here, we disclose such a probe, which, developed based on an acetyl benzocoumarin fluorophore, can be excited at 900 nm under two-photon excitation and emit in the red region. The probe shows high reactivity, selectivity, and sensitivity in *in vitro* assays. Two-photon microscopic imaging of H_2S in HeLa cells aided by the probe demonstrates that it is potentially useful to study H_2S level changes in cells and tissues influenced by external stimuli.

Introduction

Hydrogen sulfide (H_2S), the third endogenous gasotransmitter after carbon monoxide and nitric oxide,[1] regulates several physiological processes including modulation of neuronal activity, muscle relaxation, controlling insulin release, suppression of inflammation and cell protection against oxidative stress.[2] Recent studies reveals that H_2S also induces longevity and may acts as intrinsic anti-aging agent.[3] On the contrary, a few reports also mentioned that a high level of H_2S in cancer cells may be a possible target of anticancer drugs.[4] In spite of the diverse biological roles displayed by H_2S, little is known about its physiological and pathological mechanisms mainly due to the lack of reliable detection tools. Several analytical methods are developed for measurement of H_2S, including electrochemical, potentiometric, polarographic, and coloumetric assays;[5] however, these are not suitable for real-time monitoring of H_2S in biological systems where its concentration fluctuates. For *in vivo* analysis, fluorescent probes combined with fluorescence microscopy techniques are highly promising as they enable sensitive and non-invasive detection of H_2S.[6]

Accordingly, a wide range of fluorescent probes for H_2S have been reported recently, which show good sensing properties in *in vitro* systems; however, those applicable to biological systems are still limited.[7] The fluorescent probes for H_2S have been developed mainly based on a few unique reaction characteristics of H_2S: i) reduction of azides,[8] ii) tandem nucleophilic addition,[9] and iii) demetallation of Cu(II) complexes.[10] Practically useful probes must fulfil a range of criteria such as 1) fast reactivity to measure H_2S fluctuation under physiological conditions, 2) selectivity toward H_2S over high concentrations of other biological thiols and anions, 3) sensitive to detect endogenous H_2S, 4) linearly responsible within physiological H_2S concentration range, 5) biocompatible (cell permeability, intracellular stability, and low toxicity) as well as 6) capable of bioimaging by avoiding autofluorescence from biomolecules. Still, it remains a challenging task to address all of these issues.

For tissue imaging applications, additional issues such as severe light scattering, limited light penetration, autofluorescence from intrinsic biomolecules, and photobleaching of the probe should be addressed. Such issues can be alleviated to some extent by two-photon imaging under excitation in the low-energy, near-infrared wavelength region.[11] Recently, we reported a two-photon fluorescent probe (**P3**) by improving the selectivity, sensitivity, and reactivity to a practically useful level.[12] The probe belongs to a category of probes that sense H_2S through an "assisted (by formyl group)" Michael addition to an α,β-unsaturated carbonyl group.[9b] We also delineated a correct sensing mechanism, that is, a Michael addition followed by aldol condensation, rather than the early reported mechanism in related type of probes which involves double sulfide addition to the formyl and α,β-unsaturated carbonyl groups. The reactive moiety in the probe was optimized using a computational approach, leading to the di(methoxy)-substituted benzaldehyde system that showed increased steric hindrance on the Michael addition of thiols to the enone moiety as well as enhanced electrophilicity. As a result, the probe selectively responds only to H_2S/HS^- among other biothiols including glutathione or cysteine. The probe detects H_2S fast and selectively, with very high sensitivity (detection limit of

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