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Anal. Chem., Just Accepted Manuscript • DOI: 10.1021/acs.analchem.7b00073 • Publication Date (Web): 27 Mar 2017

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Ratiometric Visualization of NO/H$_2$S Cross-Talk in Living Cells and Tissues Using a Nitroxyl-Responsive Two-Photon Fluorescence Probe

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ABSTRACT: It is of scientific significance to explore the intricate relationship between two crucial gasotransmitters nitric oxide (NO) and hydrogen sulfide (H$_2$S), because they exert similar and interdependent biological actions within the living organisms. Nevertheless, visualization of the NO/H$_2$S crosstalk using effective molecular imaging tools remains challenging. To address this issue, and given that nitroxyll (HNO) has been implicated as the interdependent production of NO and H$_2$S via a network of cascading chemical reactions, we herein design a ratiometric two-photon fluorescent probe for HNO, termed TP-Rho-HNO, which consists of benzo[h]chromene-rhodol scaffold as two-photon energy transfer cassette with phosphine moiety as specific HNO recognition unit. The newly proposed probe has been successfully applied in ratiometric two-photon bioimaging of endogenous HNO derived from NO and H$_2$S interaction in the human umbilical vein cells (HUVECs) and as well as in rat brain tissues. Intriguingly, the imaging results consistently demonstrate that the mutually dependent upgeneration of H$_2$S and NO are present in living biosystems, indicating that this molecular probe would provide a powerful approach to elucidate the chemical foundation for the anfractuous cross-talk between the NO and H$_2$S signaling pathways in biology.
INTRODUCTION

Derived from its discovery as the endogenous blood-vessel “endothelium-derived relaxing factor” (EDRF), nitric oxide (NO) has been identified as a ubiquitous signaling molecule involved in a myriad of physiological as well as pathological processes in living organisms. Meanwhile, hydrogen sulfide (H₂S), historically known as a toxic pollutant, has been recently viewed as another important gasotransmitter following NO. Studies in the past few years increasingly suggest that H₂S plays crucial roles in regulating cardiovascular, neuronal, immune, endocrine, and gastrointestinal system. Interestingly, H₂S have some similar cardiovascular actions as NO including stimulation of angiogenesis, relaxation of vascular smooth muscle, and protection of ischaemia-reperfusion injury in the heart. Formerly it was generally assumed that NO and H₂S exert their biological functions via separate signaling pathways, whereas, the current evidences show that the effects of the two gasotransmitters are intimately intertwined. As a consequence, it is of scientific interest to explore the interaction between H₂S and NO in living organisms for fully elucidating the physiological and pathophysiological mechanisms via reliable and effective molecular tools.

Among the various available detection methods, fluorescence technique combining synergistic advance in probe design and imaging instrumentation is the promising one for in situ monitoring biologically important species of living organisms in real time with high spatiotemporal resolution. To date, prominent progresses have been gained in the development of fluorescent probes for NO and H₂S, respectively.
Nevertheless, it still is a virgin territory that rational design of fluorescent probe for illuminating the intricate relationship between NO and H₂S in vivo hitherto. Accumulating evidences suggest that the interaction between NO and H₂S in mammals could give rise to generation of the nitroxy (HNO) intermediate, a one-electron-reduced and protonated analogue of NO.¹¹⁻¹³ In addition, latest research proposes that the cooperative regulation of these two gasotransmitters on cardiovascular systems may attribute to the endogenous formation of the new biological mediator HNO for activating a TRPA1–CGRP signalling pathway.¹⁴ Enlightened by these reports, we thought that investigation on NO/H₂S crosstalk in vivo may be achieved by a robust HNO-responsive fluorescent probe.

Pioneered report by Lippard et al,¹⁵ several Cu(II)-based fluorescent probes for HNO had been designed through HNO-induced reduction.¹⁶⁻¹⁸ Alternatively, Nakagawa initiated metal-free ones using specific reaction between HNO and phosphine.¹⁹⁻²³ Despite of the above-mentioned advance, none of them would be ideal candidate as molecular imaging tool for visualizing the interaction between NO and H₂S in vivo owing to the following drawbacks: firstly, the metal coordination based ones might be liable to be disturbed by abundant biological reductants including glutathione and ascorbate in living systems.²⁴ Secondly, most ones rely on single-emission intensity changes, which appear to be readily influenced by external factors, including variations in excitation intensity, signal collection efficiency, local probe concentrations, and environmental conditions.²⁵ Finally, most ones have been one-photon-excited at short wavelengths, rendering them difficult to be employed in
living biological systems, because easy scattering, well absorbance, and strong
autofluorescence of biomolecules in cells and tissues elicits annoying background
interference and shallow penetration depth.\textsuperscript{26}

To address these issues and as a continuation of our researches, we sought to the
resolving method through combinating the advantages of two-photon microscopy
(TPM) and energy transfer-based ratiometric imaging approach.\textsuperscript{27,28} On the one hand,
owing to the simultaneous absorption of two photons located in the near-infrared
(NIR) region, TPM features minimal fluorescence background, increased penetration
depth of tissues, reduced photo-bleaching of fluorophores, and less photodamage to
biological sample.\textsuperscript{29,30} On the other hand, energy transfer-based ratiometric mode,
through determining the variations of the intensity ratio at two resolution emission
peaks, can provide self-calibration to minimize the aforementioned interferences and
thus enable more accurate analysis on bioimaging.\textsuperscript{31} Following this thought, Tan et al
judiciously designed the first ratiometric two-photon fluorescence probe for HNO
recently.\textsuperscript{32} Nevertheless, it is a pity that possibility of using this probe for elucidating
the NO/H\textsubscript{2}S cross-talk \textit{in vivo} was not explored.

Keeping this in mind, herein we construct a HNO-responsive ratiometric
two-photon fluorescence probe based on benzo[h]chromene-rhodol dayds, termed
\textbf{TP-Rho-HNO}, for exploring the NO/H\textsubscript{2}S cross-talk \textit{in vivo} for the first time, by
virtue of two-photon excitation fluorescence resonance energy transfer (TP-FRET)
approach. As shown in Scheme 1, under two-photon excitation condition,
\textbf{TP-Rho-HNO} only shows characteristic emission of two-photon fluorophore
benzo[h]chromene derivative because the closed spirolactone form of the rhodol
decorated with phosphine group switches off the TP-FRET process. However, in the
presence of HNO generated by interaction of NO and H₂S, the TP-FRET process
from benzo[h]chromene derivative to rhodol dye would be occurrence due to the
uncage of the recognition unit by HNO, resulting in the appearance of emission peak
of rhodol fluorophore concomitant with disappearance of that of benzo[h]chromene
derivative. Experimental results demonstrated that TP-Rho-HNO displayed selective
and sensitive response toward HNO with two-photon excited dual emission read-out,
and could ratiometrically visualize exogenous HNO in living cells and tissues at
depths by TPM. What is more, this proposed probe was successfully applied for
mapping the NO/H₂S cross-talk in the human umbilical vein cells and as well as in rat
brain tissues for the first time.

EXPERIMENTAL SECTION

Synthesis of TP-Rho-HNO. Scheme 2 depicted the synthetic procedure of
TP-Rho-HNO. Benzo[h]chromene derivative TP and rhodol derivative Rho were
firstly synthesized according to the literature methods.³³,³⁴ Subsequently, amidation
reaction of compound TP and Rho was carried out in the presence of EDC and
catalytic amount DMAP to give the two-photon energy transfer scaffold TP-Rho. The
final probe molecule TP-Rho-HNO was prepared by decorating TP-Rho with a
(diphenylphosphino)-benzoate moiety. Details for synthesizes and characterization of
all the compounds are attached to the Supporting Information.

Spectrophotometric Experiments. Test solutions were prepared by mixturin
TP-Rho-HNO and appropriate analyte stock into a tube and then diluting the solution to 500μL with 20 mM PB solution (containing 20 % acetonitrile, pH 7.4). After incubation at room temperature for 20 min, the absorption or fluorescence spectra measurements were then performed. The one-photon fluorescence spectra were recorded at emission wavelength ranging from 420 to 650 nm with excitation wavelength of 370 nm. The two-photon fluorescence emission spectra were acquired by recording with a DCS200PC single photon counting using a mode-locked Ti: sapphire pulsed laser (exciting the samples at 750 nm).

**Ratiometric TPM Imaging in Living Cells and Tissues.** For imaging of exogenous HNO in living cells, the HeLa cells were stained with 5.0 μM TP-Rho-HNO for 30 min before washed three times with PBS, then incubated with 500 μM Angeli’s salt (AS, a commercial HNO donor) solution for another 30 min. In order to monitor the intracellular crosstalk between NO and H2S, human umbilical vein cells (HUVECs) were stained with 5.0 μM probe for 30 min under different conditions: (a) the control one; (b) simultaneous incubation with 500 μM NaHS and 500 μM EDA-NONOate solutions for 1h; (c) the cells were firstly stimulated with 1 mM α-lipoic acid to promote GSH levels (used as HNO scavenger), and then incubated with 500 μM NaHS and 500 μM EDA-NONOate for 1 h; (d) incubation with 500 μM NaHS solution only for 1h; (e) incubation with 500 μM EDA-NONOate solution only for 1 h; (f) in the presence of NO scavenger 2-(4-carboxyphenyl)-4,4,5,5-tetramethyl-imidazoline-1-oxyl-3-oxide (PTIO, 500 μM), the cells were pre-treated with 500 μM NaHS for 1 h; (g) in the presence of H2S.
synthesis inhibitor phorbol myristate acetate (PMA, 1 μg/mL), the cells were pre-treated with 500 μM EDA-NONOate for 1 h. After rinsing with PBS buffer, these cells were exposed to imaging experiment on the two photon confocal microscope.

For TPM tissue imaging, the rat liver tissue slices were pre-incubated with 20.0 μM probe for 2 h, and then treated with 2 mM AS for 1h. In order to identify the endogenous HNO produced by the crosstalk between NO and H₂S in the tissues, six groups of rat brain slices were stained with 20 μM probe under different conditions for 1 h: (a) control; (b) treatment with 1 mM N-methyl-D-aspartic acid (NMDA), (c) incubation with 1 mM NaHS solution; (d) incubation with both NMDA and NaHS solution; (e) in the presence of 200 μM N-(G)-nitro-L-arginine methyl ester (L-NAME), the slice was treated with 1 mM NaHS solution; (f) in the presence of 500 μM propargylglycine (PAG), the slice was incubated with 1 mM NMDA. Procedures of imaging operations for tissue slices were similar as cellular ones. Two-photon imaging patterns were obtained from the blue channel (465-485 nm) and green channel (520-560 nm) with two-photon excitation wavelength of 750 nm. The ratiometric imaging patterns were analyzed by Image J software.

RESULTS AND DISCUSSION

Construction and Spectral Properties of Two-Photon Energy Transfer Cassette.

To design an available TP-FRET based ratiometric fluorescent probe, it is a prerequisite to construct an effective energy transfer platform which consists of a two-photon fluorophore as energy donor, a chromophore as energy acceptor, and an appropriate linker of energy transfer dyads. On the one hand, the absorption of the
acceptor should have reasonable spectral overlap with the emission of the two-photon fluorophore, on the other hand, the distance between the donor and acceptor depended on the linker should be appropriate for efficient TP-FRET to occur. Besides, the energy transfer platform should be handily modified by specific recognition unit to modulate the energy transfer efficiency for switching the emission of two-photon fluorophore.

With these in mind, we firstly fabricate a two-photon energy transfer cassette TP-Rho via hybrid of benzo[h]chromene derivative and rhodol fluorophore. Benzo[h]chromene derivative TP serves as the energy donor since it exhibits significant two-photon fluorescence properties as well as excellent photo-stability,\textsuperscript{35} which had been used to construct two-photon fluorescence bio-sensing platform by our group.\textsuperscript{36} Rhodol derivative Rho was chosen as the candidate for energy donor based on the following reasons: First, the absorption of Rho have reasonable spectral overlap with the emission spectrum of TP (Figure S1). Second, the piperazine group of Rho with rigid and short structure characteristic, playing the linker role, would be favorable for energy transfer cassette.\textsuperscript{37} Finally, rhodol could be decorated with phosphine as recognition unit for HNO.\textsuperscript{19}

After synthesis and structural characterizations of the TP-Rho (for details, see Supporting Information), we then investigate its spectral properties. As illustrated in Figure 1A, the absorption spectra of TP-Rho shows maxima at 378 and 480 nm, characteristic of the donor and acceptor constituents, respectively, meaning that the energy transfer dyads have little or no electronic interactions in the ground state.
Under two-photon excitation ($\lambda_{ex} = 750$ nm, Figure 1B), TP-Rho fluoresced with a maximum at around 540 nm, no emission band centered at 470 nm of donor component TP can be observed. In addition, the fluorescence intensity of TP-Rho is 2.7-fold brighter than that of the acceptor dye Rho upon the same irradiation condition. These results demonstrate that two photon energy transfer from donor TP to acceptor Rho take place (energy transfer efficiency is about $97.8\%$). Superior energy transfer efficiency of TP-Rho and significant distinctions of two-photon emission spectra between TP-Rho and TP imply the possibility of constructing effective ratiometric two-photon fluorescent sensing approach.

**Sensing Performances of TP-Rho-HNO.** With the two-photon energy transfer cassette TP-Rho in hand, we next prepared the HNO-responsive probe TP-Rho-HNO by decoration of TP-Rho with a (diphenylphosphino)-benzoate moiety. Following, we investigated the spectral response of TP-Rho-HNO toward HNO. As shown in Figure 2A, TP-Rho-HNO displays more apparent emission band centered at 470 nm rather than the acceptor’s characteristic emission, indicating that decoration of TP-Rho with recognition unit would lead to the closed spirolactone form to switch off the TP-FRET process. However, it's emission intensity at 470 nm gradually attenuated after treatment with increased concentrations of AS, concomitant with the appearance and drastic enhancement of fluorescence emission peak at 540 nm. This is because the electrophilic attack of HNO towards the (diphenylphosphino)-benzoate moiety of TP-Rho-HNO resulted in recovery of TP-Rho with the conjugated fluorescent xanthene form, thereby leading to an
increase in absorption of acceptor (Figure S2) as well as the occurrence of TP-FRET process. The response mechanism was precisely clarified by MS data that a new peak at m/z 651.3 of TP-Rho-HNO treated with HNO are in a well agreement with that of TP-Rho (Figure S3). In addition, the response performance of TP-Rho-HNO toward HNO was further supported by density functional theory (DFT) calculations. The calculation results are shown in Figure S4. For TP-Rho, the HOMO-LUMO energy gap of the rhodol moiety (ΔE = 2.70 eV) is less than that of the benzo[h]chromene derivative moiety (ΔE = 3.72 eV), facilitating the intramolecular energy transfer from benzo[h]chromene to rhodol. However, for TP-Rho-HNO, the energy gap of the energy acceptor (ΔE = 4.89 eV) is greater than that of the energy donor (ΔE = 3.73 eV), thus the FRET process would be suppressed.\textsuperscript{38}

Figure 2B displays that the signal to background ratio, \( S/B = \left( \frac{F_{540}}{F_{470}} \right)_n / \left( \frac{F_{540}}{F_{470}} \right)_0 \), varied from 1 to 31 with the concentration of AS changing from 0 to 400 μM, where the terms of \( \left( \frac{F_{540}}{F_{470}} \right)_n \) and \( \left( \frac{F_{540}}{F_{470}} \right)_0 \) take into account the ratios of donor and acceptor emission intensities of the probe with and without AS, respectively. Moreover, inset of Figure 2B depicted that there was a well linear relationship between the \( S/B \) and the concentrations of AS ranging from 0 to 20.0 μM with a detection limit (3σ/slope) of 50 nM, manifesting that TP-Rho-HNO is potentially adaptive for quantitative determination of trace HNO concentrations in a ratiometric manner.

The real-time fluorescence response of probe TP-Rho-HNO toward AS was examined by recording the variations in the intensity ratio of the two emission
wavelengths (F$_{540}$/F$_{470}$) after the addition of AS in PB solution. As illustrated in Figure 2C, in the absence of AS solution, TP-Rho-HNO kept silent during 25 min. By contrast, the fluorescence intensity ratio (F$_{540}$/F$_{470}$) observably enhanced and reached a plateau at around 15 min after treatment with 200 μM AS, demonstrating that the probe TP-Rho-HNO can afford a quick response toward HNO.

It is of great importance for a bioimaging probe with highly specific response to the target molecule over other potentially competing species in the complicated biosystems. Therefore, evaluation on selectivity of TP-Rho-HNO was carried out by treatment with various biologically relevant species, including bio-related metal ions, reactive oxygen species, reducing agents, small molecule thiols, and other reactive nitrogen species. As shown in Figure 2D, apart from AS, no obvious variations in S/B were observed in the presence of these interference species. These results demonstrate that the probe TP-Rho-HNO shows excellent selectivity for HNO and possesses the ability to suitably detect HNO in complicated biological environments. Moreover, TP-Rho-HNO is stable and could respond toward HNO at a biologically relevant pH level (Figure S5).

**Two Photon Ratiometric Imaging of HNO in Living Cells and Tissues.** The promising response behaviors of TP-Rho-HNO toward HNO in vitro, together with superior two-photon excitation action cross-section (Φδ) values of TP-Rho-HNO and its reaction product with AS (Figure S6), encourage us to explore whether proposed probe could function in living systems. Before bioimaging experiments, the cytotoxicities of TP-Rho-HNO against HeLa cells were primarily evaluated by
standard MTT assay (Figure S7). After cells were treated with TP-Rho-HNO (0–10 μM) for 24 h, a high cell survive rate ( > 90%) can be found. The low cytotoxic effect of the proposed probe is favorable for bioimaging application.

Subsequently, TPM imaging experiments of living cells were carried out on confocal laser scanning microscopy. As described in Figure S8, HeLa cells incubated with 5.0 μM TP-Rho-HNO show fluorescence in blue channel (465-485 nm), but there is negligible fluorescence signal through green channel (520-560 nm). On the contrary, the probe-loaded cells appear clear cellular profiles with bright green fluorescence when treated with 200 μM AS for 30 min, the original fluorescence signal in the blue channel weakened accordingly. These imaging results indicate that TP-Rho-HNO shows pleasurable cell-permeability and could be applied in dual-color fluorescence imaging of intracellular HNO.

To further interrogate the capability of TP-Rho-HNO in fluorescence imaging of living systems, two-photon ratiometric imaging of TP-Rho-HNO for exogenous HNO in rat liver tissue slices was then performed by TPM. As shown in Figure S9, the tissue stained with probe only showed fluorescence signal in blue channels (465-485 nm). Moreover, Figure S10 revealed that the fluorescence signals were collected at different tissue depths (0–200 μm) using Z-scan mode, indicative of the applicability of TP-Rho-HNO for deep-tissue imaging. In contrast, relatively obvious fluorescence could be observed in the green channel after incubation with AS, concomitant with a disappearance of fluorescence signal in the blue channel. These results demonstrate that TP-Rho-HNO possesses predominant tissue penetration and
ratiometric imaging performance.

**Two Photon Ratiometric Imaging of H$_2$S/NO Cross-talk in Living Cells and Tissues.** Given that HNO has been implicated as the interdependent production of the two gasotransmitters NO and H$_2$S via a network of cascading chemical reactions and encouraged by the above promising ratiometric imaging results of TP-Rho-HNO, we turn our attention to further interrogate its feasibility to visualize H$_2$S/NO cross-talk by determination of endogenous HNO in living systems.

Initially, the human umbilical vein cells (HUVECs) were treated with NaHS (H$_2$S donor) and DEA-NONOate (NO donor) in the absence and presence of PMA (H$_2$S synthesis inhibitor) and PTIO (NO scavenger) for cellular imaging experiments (Figure 3). In comparison with the free TP-Rho-HNO loaded HUVECs (Figure 3A), there was significantly incremental fluorescence in green channel concomitant with signal attenuation in blue channel for the probe-treated HUVECs with the presence of both NaHS and DEA-NONOate, leading to highlighted fluorescence ratio signal change (Figure 3B). However, Figure 3C showed that, even though simultaneous treatment with NaHS and DEA-NONOate solutions, fluorescence change remained slight when probe-loaded HUVECs were pre-incubated with α-lipoic acid (GSH stimulator), consistent with HNO could being consumed by excess glutathione (GSH). It manifest that TP-Rho-HNO could be suitable for visualizing endogenously generated HNO from interplay between the two gasotransmitters H$_2$S and NO inside HUVECs. Unexpectedly, we note that a certain of fluorescence ratio change could be found from probe-loaded cells in the presence of NaHS solution...
alone (Figure 3D). Intriguingly, similar image patterns had been achieved for DEA-NONOate-treated HUVECs (Figure 3E). Therefore, it is hypothesized that endogenous H$_2$S and NO have been irritatingly produced by their opposite gasotransmitter. To further interrogate this mutually dependent process, two group control HUVECs were pre-incubated with PTIO and PMA as the scavengers of NO and H$_2$S synthesis inhibitor, individually.$^{42,43}$ Authentically, the corresponding ratiometrical fluorescence changes disappeared when dealing with NaHS or DEA-NONOate solutions respectively (Figure 3F, 3G). In addition, H$_2$S-stimulated NO production and vice versa were further verified by using DAF-FM DA fluorescence dye (a commercial NO probe$^{44}$) and a H$_2$S-specific two-photon probe previously reported by us$^{45}$ (Figure S11, S12). These combined imaging experiments definitely confirm that endogenous H$_2$S and NO generation could be upregulated with each other undoubtedly, in accord with previously reported studies.$^{46,47}$

We proceeded to evaluate the capability of TP-Rho-HNO for visualizing endogenous HNO derived from H$_2$S and NO interaction inside living brain tissues. As we known, NO is exclusively produced from L-arginine in a series of reactions catalyzed by nitric oxide synthase (NOS).$^{48}$ Moreover, it is approved that endogenous H$_2$S formation is mainly dependent on cystathionine beta-synthase (CBS) in the brain.$^{49}$ Therefore, TPM tissue imaging experiments were performed through regulating enzymatic NO and/or H$_2$S production to determinate the endogenous HNO fluctuations at depths in fresh slice of rat brain. As depicted in Figure 4B that, distinction from the free probe-stained slice (Figure 4A), quiet a few fluorescence in
the green channel emerged along with slight signal attenuation in the blue channel for
the probe-stained slices in the presence of N-methyl-D-aspartic acid (NMDA), a
reagent as NOS activator,\textsuperscript{50} indicating that not only NO but also H\textsubscript{2}S are
enzymatically generated, consistent with the previous research that NO stimulates
CBS activity to result in endogenous H\textsubscript{2}S production.\textsuperscript{51} Likewise, a certain change of
ratiometric pattern could be observed for NaHS treated-slice (Figure 4C). It implicates
that, similar as the above results of cellular level, interdependent formations of the
two gasotransmitters are also constitutively present in the tissue samples. What is
more, the fluorescence signals of a contrast sample changed dramatically in
bule/green dual channels upon simultaneous treatment with NaHS and NMDA
solution, bringing about considerable enhancement of fluorescence ratio signal
(Figure 4D). In sharp contrast, as illustrated in Figure 4E and 4F, regardless of the
presence of endogenous H\textsubscript{2}S or stimulation endogenous NO production, fluorescence
ratio signals of the control ones attenuated strikingly when pre-dealing with either
L-NAME (inhibitor of NOS)\textsuperscript{50} or PAG (a commercial inhibitor of CBS)\textsuperscript{52},
correspondingly. Taken together, these imaging findings unambiguously reveal that
HNO formation in tissues indeed originates from the interaction of enzymatically
generated H\textsubscript{2}S and NO.

**CONCLUSION.** In summary, we have constructed a HNO-responsive two-photon
ratiometric fluorescence imaging probe based on benzo[h]chromene-rhodol dayds for
exploring the cross-talk between H\textsubscript{2}S and NO in vivo for the first time. This probe,
designed by TP-FRET process, exhibits striking blue-to-green fluorescence emission
change in response to HNO with excellent characters including sensitivity, rapid response, positive selectivity, and robust staining ability of living cells and tissues, thereby allowing ratiometrical visualization of endogenous HNO in living systems. Above all, TPM imaging results not only consistently demonstrated the pathway of endogenous HNO formation attributed to H$_2$S and NO interaction, but also clearly revealed the interdependent upgeneration of H$_2$S and NO within cells and brain tissue, indicating that this newly designed probe would be an available molecule tool to elucidate the chemical foundation for the anfractuous cross-talk between the NO and H$_2$S signaling pathways in biology.

ASSOCIATED CONTENT

Supporting Information. The supporting information is available free of charge via the Internet at http://pubs.acs.org. Experimental details, synthesis, NMR and mass spectra, and additional spectroscopic data as noted in text.

ACKNOWLEDGMENTS

We are grateful to professor Zhihong Liu and Dr Zhiqiang Mao of Wuhan University for their selfless assistance about the measurement of two photon excitation spectra. This work was financially supported by the National Natural Science Foundation of China (21505006, 21575018, 21605008), the Foundation for Innovative Research Groups of NSFC (21521063), the Scientific Research Fund of Hunan Provincial Education Department (16C0033,16C0032), and the Open Fund of State Key Laboratory of Chemo/Biosensing and Chemometrics of Hunan University (2015011,2015003).
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Scheme 1. Response Mechanism of TP-Rho-HNO toward HNO for Exploring the NO/H₂S Cross-Talk.
Scheme 2. Synthetic Procedure of TP-Rho-HNO.
Figure 1. (A) UV-vis absorption spectra of **TP** (a, 10.0 μM), **Rho** (b, 10.0 μM), and **TP-Rho** (c, 10.0 μM) in PB solution (pH = 7.4, containing 20 % MeCN). (B) Two-photon excitation fluorescence spectra of **TP** (a, 2.0 μM), **Rho** (b, 2.0 μM), and **TP-Rho** (c, 2.0 μM) in PB solution (pH = 7.4, containing 20 % MeCN). $\lambda_{ex} = 750$ nm.
Figure 2. (A) One-photon fluorescence response of TP-Rho-HNO (2.0 μM) to AS (0–400.0 μM) in PB solution (pH = 7.4, containing 20 % MeCN). λ<sub>ex</sub> = 370 nm. (B) Signal-to-background ratios (S/B) of TP-Rho-HNO as a function of AS concentrations (0–400 μM) in PB solution (pH = 7.4, containing 20 % MeCN). Inset: linear responses of S/B of TP-Rho-HNO to changing AS concentrations (0–20 μM). (C) Real-time records for fluorescence ratio (F<sub>540/F<sub>470</sub>) changes of TP-Rho-HNO in the absence and presence of 200.0 μM AS solution. λ<sub>ex</sub> = 370 nm. (D) S/B changes of TP-Rho-HNO for AS in the presence of other common species in PB solution (pH = 7.4, containing 20 % MeCN). The concentrations of AS and other species were 400 μM and 1 mM, respectively.
Figure 3. Two-photon ratiometric fluorescence images of NO/H$_2$S crosstalk in HUVECs using 5.0 μM TP-Rho-HNO under different conditions: (A) the probe only. (B) in the presence of 500 μM NaHS and 500 μM DEA-NONOate. (C) in the presence of 500 μM NaHS and 500 μM DEA-NONOate before pre-incubation with 500 μM α-lipoic acid. (D) in the presence of 500 μM NaHS. (E) in the presence of 500 μM DEA-NONOate. (F) the cells was pre-treated with PTIO, further incubated with 500 μM NaHS. (G) the cells was pre-treated with PMA, further incubated with 500 μM DEA-NONOate. The images were collected at 465-485 nm (blue channel) and 520-560 nm (green channel) upon excitation at 750 nm. Scale bar: 20 μm.
**Figure 4.** Two-photon ratiometric fluorescence images of crosstalk of NO and H$_2$S in fresh rat brain tissues at the depth of 100 μm with 40× magnification using 20 μM **TP-Rho-HNO** under different conditions: (A) the probe only; (B) 1 mM NMDA; (C) 1 mM NaHS; (D) 1 mM NMDA + 1 mM NaHS; (E) 200 μM L-NAME + 1 mM NaHS. (F) 500 μM PAG + 1 mM NMDA. The images were collected at 465-485 nm (green channel) and 520-560 nm (red channel) upon excitation at 750 nm. Scale bar: 50 μm.
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