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A ratiometric Raman probe for live-cell imaging of hydrogen sulfide in mitochondria by stimulated Raman scattering

Chen Zeng, Fanghao Hu, Rong Long, and Wei Min

Department of chemistry, Columbia University, 3000 Broadway, New York, NY 10027, USA.
wm2256@columbia.edu

Abstract

Stimulated Raman scattering (SRS) coupled with alkyne tags has been an emerging imaging technique to visualize small-molecule species with high sensitivity and specificity. Here we describe the development of a ratiometric Raman probe for visualizing hydrogen sulfide (H_2S) species in living cells, as the first alkyne-based sensor for SRS microscopy. This probe uses an azide unit as a selective reactive site, and it targets mitochondria with high specificity. The SRS ratiometric images display strong response to H_2S level changes in living cells.

Graphical Abstract

We develop a ratiometric Raman probe for visualizing hydrogen sulfide in living cells, as the first alkyne-based sensor for SRS microscopy.

Raman microscopy has been a powerful technique for imaging biomolecules in cells. It can detect the intrinsic vibration of chemical bonds in the molecules and doesn't require exogenous labeling.^{1, 2} However, the label-free approach has the limitation of overlapping Raman spectra and it is difficult to distinguish the target molecule from other endogenous biomolecules when they share the same chemical bond. Therefore, there is a high demand for developing Raman tags to overcome the limited molecular specificity. Several functional groups have been developed as Raman tags, including alkyne, nitrile, and carbon deuterium bond.^{3–7} Alkynes show strong Raman intensity compared to other tags and possess a sharp

Conflicts of interest

There are no conflicts to declare.

Electronic Supplementary Information (ESI) available: [details of any supplementary information available should be included here].

Raman peak in the cell-silent Raman spectral window (1800–2600 cm^{-1}), where there is negligible endogenous Raman background from other biomolecules in the cells. Besides, alkyne is bioorthogonal, which make it a suitable tag for Raman microscopy in live cells.

Stimulated Raman scattering (SRS), as an emerging nonlinear optical imaging technique, enhances the otherwise weak Raman transition by a factor of 10^8 and improves the imaging speed by 100–1000 times over spontaneous Raman microscope.^{8–11} In addition, SRS intensity is linearly dependent on the concentration of the chemical bond, making it very useful for quantitative analysis. In 2014, metabolic incorporation of alkyne-tagged small biomolecules has been developed for visualizing the newly synthesized DNA, RNA, proteins and lipids in living cell by SRS microscopy.^{12, 13} Since then, SRS microscopy combined with the alkyne tags have been applied to study glucose metabolism,^{14, 15} cholesterol metabolism,¹⁶ multicolor imaging^{17, 18} and mitochondrial imaging.¹⁹ Very recently, polyne-based probes were engineered to achieve ten-color optical imaging of organelles in living cells.²⁰ Although the combination of SRS microscopy with alkyne tags has been proven useful for imaging various metabolites and organelles, there is no report yet on the sensing of small molecules or ions using this technique in living cells. The present paper would report the first demonstration of such applications.

Hydrogen sulfide (H_2S), a well-known pungent gas, is a vital gaseous signaling molecule related to many physiological processes.^{21, 22} Endogenous H_2S in mammalian cells is mainly produced from cysteine and homocysteine, catalyzed by enzymes including cystathionine- γ -lyase (CSE), 3-mercaptopyruvate sulfurtransferase (MST), and cystathionine β -synthase (CBS). The production can occur in both the cytosol and mitochondria, where the enzymes locate primarily.²³ The physiological H_2S levels in mammalian cells have been reported in the range from 0.1 μM to 300 μM . Abnormal H_2S levels has been correlated to many diseases such as Alzheimer's disease,²⁴ Down's syndrome,²⁵ diabetes,²⁶ and liver cirrhosis.²⁷ Therefore, it is of great importance to detecting H_2S in living cells. Many fluorescent probes have been reported for imaging H_2S .^{28–35} However, fluorescence imaging suffers from background signals, photobleaching and limited resolvable spectra, which would hinder simultaneous multicolor detection of different small molecule species in living cells. Here, we developed an alkyne-based Raman probe to visualize H_2S in living cells by SRS microscopy. This Raman probe shows negligible background, non-photobleachable, and minimal phototoxicity. Moreover, owing to the sharp Raman resonance, this probe can be potentially combined with other Raman probes to reach super-multiplex sensing of a large number of different small molecule species within single cells, in a manner similar to the newly demonstrated super-multiplex organelle imaging in living cells.²⁰ Such a potential super-multiplexing ability is the unique advantage of Raman probes over the conventional fluorescent probes.

We designed our Raman probe **1** based on the well-established reaction of aromatic azide with H_2S .²⁸ The azide group could be reduced to amine group by H_2S selectively to form **2** (Fig. 1). In addition, a diyne scaffold with aryl end-capping groups (2-yne) was applied as a specific Raman reporter because of the strong intensity in the cell-silent Raman region. Due to the significant change in the electronic properties,²⁰ we hypothesize that the Raman frequency of product **2** would red shift compared to the probe **1**, since azide is an electron-

withdrawing group while amine is a strong electron-donating group. The positive-charged triphenylphosphium (TPP) group was further incorporated to target mitochondria through the high affinity to the negatively-charged mitochondrial matrix.³⁶

As shown in Scheme 1, our synthesis commenced with commercial available 4-ethynylaniline **3**, which was converted into 4-ethynylphenyl azide **4** by diazo-transfer method using 2-azido-1,3-dimethylimidazolium hexafluorophosphate (ADMP) as the reagent.³⁷ Glaser–Hay coupling of **4** with 4-ethynylbenzyl alcohol provided 2-yne **5**, which was coupled with **6** containing a TPP group by a carbamate bond to afford the Raman probe 2-yne Mito N₃ **1**. The 2-yne Mito N₃ **1** could be transformed into 2-yne Mito NH₂ **2** by NaSH in DMSO/H₂O solution and demonstrate the feasibility of our Raman probe **1** for detecting H₂S. Both **1** and **2** display good chemical stability and photostability.

With **1** and **2** in hand, we measured both the Raman spectra by SRS microscopy (Fig. 2a). Both of them show a sharp peak in the cell-silent Raman region and the vibrational frequency shifts by 9 cm⁻¹, from 2223 cm⁻¹ for 2-yne Mito N₃ to 2214 cm⁻¹ for 2-yne Mito NH₂, which is qualitatively consistent with our hypothesis. In the UV-Vis absorption spectra (Fig. 2b), we also observed a red shift in the longest wavelengths of absorption as the substituent changes from N₃ to NH₂ with increased electron density. Furthermore, we measured the SRS sensitivity of **1** at 2223 cm⁻¹ at different concentrations in DMSO solutions using SRS microscopy, which gives a detection limit of 15 μM with a signal-to-noise ratio of 1 at a 100 μs time constant (Fig. S1).

We then studied the reaction kinetics of our Raman probe **1** with NaSH (a commonly used H₂S donor) in phosphate buffered saline (PBS) solution (Fig. 2c). 50 μM **1** was treated with 500 μM NaSH in PBS for different time (0, 15, 30, 60, 90 and 120 min) and SRS intensity was measured at 2214 cm⁻¹ and 2223 cm⁻¹. The ratio of SRS intensity (2214 cm⁻¹/ 2223 cm⁻¹) gradually increases from 0.37 at 0 min to 2.01 at 60 min and reaches to maximum 2.30 at 90 min, which is very close to the ratio value of pure product **2**. We also monitored the reaction of the probe **1** with NaSH by mass spectrometry (Fig. 2e). The mass peak of reactant **1** is at 619.5 and that of the product **2** lies at 593.5. Addition of NaSH to **1** led to significant increase of the product peak, which became the major peak at 30 min. There is only a tiny peak at 619.5 at 60 min and no starting material peak was detected at 90 min. These data confirm that the reaction proceeds completely in less than 90 min.

Moreover, we examined the specificity of **1** for H₂S over other biologically-relevant reactive sulfur, oxygen, and nitrogen species. The ratio of the SRS intensity (2214 cm⁻¹/ 2223 cm⁻¹) showed 6-fold response for H₂S from 0.4 to 2.3 (Fig. 2d). On the contrary, other biologically reactive species including cysteine (Cys), glutathione (GSH), sulfite (SO₃²⁻), hydrogen peroxide (H₂O₂) and nitrite (NO₂⁻) show very minor changes. Therefore, our Raman probe **1** can selectively respond to H₂S over other species in the biological system.

Encouraged by the fast reaction kinetics and high selectivity in PBS solution, we next assessed the ability of the Raman probe for live-cell imaging. Firstly, we successfully visualized strong SRS signal in live human cervical cancer cells HeLa for both 2-yne Mito N₃ **1** at 2223 cm⁻¹ (Fig. 3a, middle) and 2-yne Mito NH₂ **2** at 2214 cm⁻¹ (Fig. 3b, middle).

SRS images at 2941 cm^{-1} (mainly protein CH_3 vibration) display distribution of the total proteins and outline the cell morphology (Fig. 3). The SRS off-resonance channels (2000 cm^{-1}) show negligible background, verifying the background-free detection of SRS microscopy. We also tested the cytotoxicity of **1** in HeLa cells and our Raman probe **1** showed minimal cytotoxicity (Fig. S2).

We next investigated the mitochondria targeting capability of our probe in two cell lines, including HeLa and monkey kidney fibroblast cells COS-7. HeLa (Fig. 4a) and COS-7 (Fig. 4b) cells were incubated with **1** ($10\text{ }\mu\text{M}$) for 1h and MitoTracker Deep Red (200 nM) for 30 min. Both the SRS channel and the fluorescence channel (excitation at 635 nm) show highly similar signals and the merged patterns show good co-localization, indicating the major distribution of **1** in mitochondria. In addition, the product **2** ($10\text{ }\mu\text{M}$) shows similar patterns in living HeLa and COS-7 cells (Fig. S3), demonstrating the retained mitochondria localization after sensing H_2S .

Lastly, we apply 2-yne Mito N_3 **1** to visualize variations of H_2S levels in live HeLa or COS-7 cells. To enhance the contrast for ratiometric imaging in live cells, 2228 cm^{-1} and 2212 cm^{-1} are optimally selected with reduced spectral overlap for the probe **1** and product **2**, respectively. Cells incubated with H_2S probe **1** ($10\text{ }\mu\text{M}$) alone show weak SRS image at 2212 cm^{-1} but strong SRS signals at 2228 cm^{-1} (Fig. 5a and 5c). Incubating cells with **1** ($10\text{ }\mu\text{M}$) for 30 min and then treated with $200\text{ }\mu\text{M}$ NaSH (a concentration comparable with physiological H_2S levels) for 1 h result in a significant increase in Raman signal at 2212 cm^{-1} and strong decrease at 2228 cm^{-1} (Fig. 5b and 5d). The ratiometric maps ($2212\text{ cm}^{-1}/2228\text{ cm}^{-1}$) provided a more direct visualization of H_2S sensing in live cells (Fig. 5, right). More quantitatively (Fig. 5e), HeLa cells treated with $200\text{ }\mu\text{M}$ NaSH exhibit a ratio of 1.58 ± 0.07 , which is about 2.5-fold higher compared to the ratio (0.64 ± 0.08) in untreated HeLa cells without addition of NaSH. Similar results are obtained in the COS-7 cells. Thus, these data demonstrate that our Raman probe **1** is able to sense the H_2S change at mitochondria in living cells.

In summary, we presented a ratiometric Raman probe for visualizing H_2S molecules in living cells by SRS microscopy. This new alkyne-based probe **1** equips with an azide functional group as reactive site and shows high selectivity to H_2S over other biologically reactive species. It also shows negligible background, non-photobleachable, and minimal phototoxicity in living cells. We demonstrated the applications of the probe for sensing H_2S change at mitochondria in live HeLa and COS-7 cells. We anticipate that this approach can be generally applied for sensing other biologically important molecules in living systems. Furthermore, with future probe development, we can potentially achieve super-multiplex sensing of different small molecule species within single cells by the combination of Raman sensors with distinct Raman frequencies. This super-multiplexing ability is the unique advantage of Raman probes over the conventional fluorescent probes.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1.
The structure and mechanism of Raman probe for sensing H_2S

Fig. 2.

(a) Raman spectra of 2-yno Mito N₃ **1** (orange) and 2-yno Mito NH₂ **2** (blue) in DMSO solutions. (b) Absorption spectra of 10 μM of **1** (orange) and **2** (blue) in DMSO solutions. (c) Time-dependent ratiometric SRS (2214 cm⁻¹/ 2223 cm⁻¹) response of the probe **1** (50 μM) with 10 equiv. NaSH at 0, 15, 30, 60, 90 and 120 min after addition of NaSH. (d) Ratiometric SRS (2214 cm⁻¹/ 2223 cm⁻¹) response of **1** (50 μM) to other biologically relevant reactive sulfur, oxygen and nitrogen species. Data were acquired at 90 min after addition of analyte (500 μM for NaSH, Na₂SO₃, H₂O₂ and NaNO₂; 5 mM for GSH and Cys). (e) Mass spectrum of 10 μM **1** with 100 μM NaSH in PBS at 0, 30, 60 and 90 min

Fig. 3. SRS imaging of **1** or **2** in living HeLa cells. (a) HeLa cells were incubated with 10 μM **1** for 30 min at 37 °C. (b) HeLa cells were incubated with 10 μM **2** for 30 min at 37 °C. Scale bar: 10 μm.

Fig. 4. Co-localization imaging of **1** in living cells. (a) HeLa or (b) COS-7 cells were incubated with 10 μM **1** for 1 h and 0.2 μM Mito Tracker Deep Red for 0.5 h at 37 °C. Scale bar: 10 μm.

Fig. 5. Ratiometric SRS images (2212 cm⁻¹/2228 cm⁻¹) in living cells. (a) HeLa or (c) COS-7 cells were incubated with 10 μM **1** for 0.5 h at 37 °C. (b) HeLa or (d) COS-7 cells incubated with 10 μM **1** for 0.5 h and then incubated with 200 μM NaSH for 1 h at 37 °C. (e) Quantitative analysis of the ratio of 2212 cm⁻¹/ 2228 cm⁻¹. Scale bar: 10 μm. Error bar: standard deviation.

Scheme 1.
Synthesis of Raman probe