HHS Public Access

Author manuscript

Analyst. Author manuscript; available in PMC 2016 February 09.

Published in final edited form as:

Analyst. 2016 February 8; 141(4): 1209–1213. doi:10.1039/c5an02497h.

Genetically Anchored Fluorescent Probes for Subcellular Specific Imaging of Hydrogen Sulfide

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Abstract

Imaging hydrogen sulfide (H_2S) at the subcellular resolution will greatly improve the understanding of functions of this signaling molecule. Taking advantage of the protein labeling technologies, we report a general strategy for the development of organelle specific H_2S probes, which enables sub-cellular H_2S imaging essentially in any organelles of interest.

Hydrogen sulfide (H_2S) is the most recently discovered member of gasotransmitters, along with nitric oxide (NO) and carbon monoxide.[1] H_2S is mainly produced by three enzymes, including cystathionine β-synthase (CBS), cystathionine γ-lyase (CSE), and 3-mercaptopyruvate sulfurtransferase (MST).[2] H_2S has been found to play many important roles in the regulation of blood pressure, cardiac response to ischemia injury, and inflammation.[2] It can also perform *S*-sulfhydration on proteins to modulate their activities.[3] It is known that the subcellular distribution of H_2S producing enzymes changes under different physiological conditions. For example, liver ischemia can lead to the accumulation of CBS from cytosol into mitochondria.[4] In addition, cytoplasmic CBS and CSE can be translocated into nucleus upon SUMO modification despite the roles of this translocation are unclear.[5] Similarly, distribution and functions of H_2S in different organelles are not well understood. It was reported that H_2S can not only regulate mitochondrial bioenergetics functions,[6] but also augment synaptic neurotransmission in the nucleus of the solitary tract.[7]

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Electronic Supplementary Information (ESI) available: Experimental procedures and characterization

Because variations in the spatial and temporal production of H_2S can induce different downstream biological effects, it is important to track H_2S with subcellular resolution.[1] In this contribution, we present a new class of organelle-specific fluorescent probes for H_2S leveraging protein labeling techniques and demonstrate its applications in cells.

Several fluorescent H_2S probes for intracellular imaging were previously developed.[8-12] The groups of Chang and Wang took advantage of the reducing potential of H_2S to convert azides to amines to achieve a "turn-on" fluorescence response. Xian et al. reported a probe containing bis-electrophilic centers for H_2S detection.[13] He et al. designed a Michael addition-based H_2S probe.[14] These probes, however, lack organelle specificity. The groups of Kim and Guo applied positively charged molecules as targeting moieties to direct H_2S probes to mitochondria,[15, 16] while Cui et al. developed a morpholine containing H_2S probe for lysosome targeting.[17] However, these approaches cannot be generalized to other organelles and to H_2S producing enzymes, such as CBS and CSE. Ai et al. developed a genetically encoded H_2S probe based on an unnatural amino acid modified green fluorescent protein, which has the potential to fuse with any subcellular locating sequences to achieve organelle specific H_2S detection.[18] The caveat for Ai's H_2S probe is currently limited to green fluorescence proteins.[19] Therefore, a generalized alternative approach for organelle specific H_2S probe with broad color palette is needed for H_2S related research.

Fluorescent labeling of specific proteins in live cells has achieved significant advancement in recent years.[20] Among these techniques, SNAP/CLIP-tag® technologies demonstrated to be reliable and applicable to wide range of target proteins. The SNAP/CLIP-tag® technology, developed by Johnsson and co-workers, is based on the O^6-alkylguanine-DNA alkyltransferase (AGT) fusion protein which, once it is fused with a protein of interest, reacts covalently with labeling molecules.[21] These protein labeling techniques have been widely applied for specific labeling of proteins in live cells[21, 22] and in animals,[23] and directing fluorescent probes to detect Ca^{2+}, Zn^{2+} and H_2O_2 in specific organelles.[24-27]

Our design of the organelle-specific fluorescent probes for H_2S is composed of an azide based H_2S fluorescent probe and a tagging substrate for SNAP/CLIP technologies (Figure 1). This strategy can be applied to any subcellular locations of interest. In this contribution, we demonstrate its applicability by developing H_2S fluorescent probes that specifically target mitochondria and for the first time – the nucleus.

We chose 7-azido-coumarin ($CouN_3$) as the H_2S probe that fluoresces with 405 nm of excitation because i) it has been established that the azide group can quench the coumarin fluorescence, and upon reacting with H_2S, azide can be converted to an amine group, which restores the fluorescence of coumarin;[28] ii) coumarin can be applied in two-photon fluorescence imaging, which facilitates its *in vivo* applications;[29] and iii) our design can potentially complement with Ai's H_2S responsive GFP in a multi-color organelle specific H_2S detection.[19] $CouN_3$ was conjugated with a O^2-benzylcytosine (BC) moiety – the CLIP-tag substrate (Figure 2A). The synthesis of the $CouN_3$-BC conjugate is described in the Supporting Information (SI).

The spectroscopic changes and reaction kinetics of $CouN_3$-BC with H_2S were investigated. $CouN_3$-BC exhibits very minimal fluorescence with an excitation wavelength at 405 nm (Figures 2B and S1). Upon reacting $CouN_3$-BC with H_2S, the fluorescence at 450 nm increases 35 folds within 1 h (Figure 2B). It should be noted that Barrios et al. reported a 4-fold increase of fluorescence intensity for a similar H_2S probe upon reacting with 100 μM of H_2S for 1 h,[28] which is ~9 times lower than the fluorescence enhancement from our observation. Based on our experiments, we found that $CouN_3$-BC is photosensitive and aged $CouN_3$-BC usually has 4-5 folds higher baseline fluorescence than the newly prepared counterpart. Therefore, all our $CouN_3$-BC samples used in the fluorescence measurements were purified with flash chromatography in the dark and used immediately. In addition, because H_2S solution is prone to oxidation in air, we carried all the experiments in Figure 2 under anaerobic conditions. We suspect that the experimental details may account for the discrepancy between our and Barrios' studies. In order to verify that $CouNH_2$-BC is the product for the reaction between $CouN_3$-BC and H_2S, we applied liquid chromatography-mass spectrometry (LC-MS) to monitor the reaction and found a new product with molecular weight that matches $CouNH_2$-BC (Figure S2).

$CouN_3$-BC is a highly specific probe for H_2S. In order to test the reaction specificity of $CouN_3$-BC towards H_2S, we incubated $CouN_3$-BC with various reductive and oxidative species for 1 h and monitored the changes of the fluorescence intensity at 450 nm. As shown in Figure 2C, the fluorescence intensity increased significantly when $CouN_3$-BC was incubated with PBS buffer containing 100 μM of H_2S. In contrast, there were minimal fluorescence changes observed when $CouN_3$-BC was incubated with other sulfur containing compounds, intracellular reductants or oxidants. Our result is consistent with previous studies using azides as a H_2S specific group[18, 30] and demonstrate the H_2S specificity of $CouN_3$-BC. It should be noted that in order to simplify this model study, $CouN_3$-BC was used instead of the conjugate of $CouN_3$ and CLIP-tag protein, which is the functional unit inside cells. Because the BC moiety has little absorbance at 405 nm, we do not expect that it interferes with the fluorescence of $CouNH_2$. Furthermore, based on Lippard's study, AGT conjugated Zn^{2+} probe has very similar reactivity to the parent probe.[24] Therefore, we believe that $CouN_3$-BC is a reasonable model compound to study the spectroscopic changes and reactivity in the presence of H_2S and other redox species.

$CouN_3$-BC is cell-permeable and can cross plasma membrane in a concentration dependent manner. In order to apply $CouN_3$-BC for live cell imaging, it should be able to permeate cells. In addition, the excess $CouN_3$-BC that has not reacted with the CLIP-tag proteins should diffuse out of the cells in the washing steps. To test the cell permeability of $CouN_3$-BC, we incubated $CouN_3$-BC with HeLa cells for 30 min, followed by the addition of H_2S for another 30 min. We observed strong fluorescence inside the cells, indicating $CouN_3$-BC can enter cells (Figure S3). When incubation was followed by PBS washing, however, subsequent addition of H_2S solution did not induce any fluorescence increase, indicating that the excess $CouN_3$-BC that does not react with the CLIP-tag protein can diffuse out of cells (Figure S3).

$CouN_3$-BC can probe the changes of H_2S levels with subcellular specificity through conjugation with CLIP-tag fusion proteins. In this "proof-of-concept" study, we chose

nucleus and mitochondria as the subcellular targets for $CouN_3$-BC because many of the known H_2S mediated biological processes are involved with these two organelles.[3, 5, 7] Two plasmids pCLIP-H2B and pCLIP-COX8A were used to transiently express CLIP fusion proteins to localize $CouN_3$-BC to nucleus and mitochondria, respectively. The pCLIP-H2B construct targets the CLIP tag to the nuclear matrix with the help of the nucleus localization sequence on the fused histone H2B protein.[31] The pCLIP-COX8A plasmid expresses cytochrome c oxidase subunit VIIIA (COX8A) with a fused CLIP tag that localizes at the inner membrane and the matrix of mitochondria.[25]

HeLa cells were transiently transfected with pCLIP-H2B and pCLIP-COX8A for 72 h using lipofectamine® 3000, and then incubated with $CouN_3$-BC to localize the H_2S probe to nucleus and mitochondria, respectively. Excess $CouN_3$-BC was removed by PBS washing. Subsequently, exogenous H_2S (250 μM) was added into the medium to induce the fluorescence responses from the H_2S probe. Secondary staining was also performed to confirm the organelle specificity of the H_2S probe. The negative control cells were transfected with control vectors. All the cells were treated with $CouN_3$-BC and H_2S, and their corresponding controls.

For the negative control cells, there was minimal fluorescence observed in the blue (405 nm) channel even in the presence of both $CouN_3$-BC and H_2S, indicating that $CouN_3$-BC can be efficiently removed if there are no CLIP-tag fusion proteins inside cells (Figure 3).

For pCLIP-H2B transfected cells, there was minimal fluorescence observed in the blue channel if either $CouN_3$-BC or H_2S was added to the cells, suggesting that there is low background from cells and $CouN_3$-BC (Figure 3). In contrast, significant fluorescence increase was observed in the nucleus *only* when cells were treated with both $CouN_3$-BC and H_2S, indicating an organelle specific H_2S response. It should be noted that these organelle-specific H_2S probes can only reflect the presence of H_2S in that organelle, but cannot determine whether H_2S is produced in the organelle due to the diffusive nature of H_2S inside cells.

In order to further confirm the organelle specificity of the H_2S probe, the cells were stained with NucRed® Live 647 nuclear stain and an Alexa 488 conjugated antibody against COX-IV,[32] cytochrome c oxidase complex as a marker of the mitochondrial inner membrane (off-target control). From the pseudo colored merged image of different channels, we can observe a substantial area in purple (overlay of the blue and red channels, Figure 3) indicating a significant accumulation of the H_2S probe in nucleus. Quantitative colocalization analyses further confirmed a significant correlation based on the Pearson's and Manders' coefficients (Table S1). We could not detect any overlap between the blue and green channels, demonstrating minimal off-target effect of $CouN_3$-BC. To the best of our knowledge, this is the first example of a nuclear specific H_2S probe.

To demonstrate the general applicability of our genetically anchored H_2S probe, a similar experiment was performed using pCLIP-COX8A treated cells. We also observed substantial co-localization (cyan in Figure 3) of the fluorescence signals from the mitochondria targeted probe and the Alexa 488 conjugated antibody against COX-IV, a mitochondrial marker

protein (Table S1). In contrast, little co-localization was observed between the blue channel (probe) and the red channel (nucleus), suggesting minimal off-target effect for the mitochondria targeted H_2S probe. It should be cautioned that the fluorescence of $CouNH_2$ can be quenched in acidic environment due to protonation of the aromatic amino group. Therefore, sensitivities of these probes may be affected if applied in endosomes or lysosomes.

The organelle specific $CouN_3$ probe is also responsive to endogenous H_2S. It is reported that pharmacological donors of NO can up-regulate cysteine uptake and CBS activity, resulting in increase of H_2S production.[33] To test whether $CouN_3$ probe is responsive to endogenous H_2S, HeLa cells were transiently transfected with pCLIP-H2B, and then incubated with $CouN_3$-BC to localize the H_2S probe to nucleus. Subsequently, cells were treated with sodium nitroprusside (SNP), an NO donor, to induce H_2S production. Consistent with previous study,[34] SNP treated cells showed significantly higher H_2S concentration in nucleus than the control cells (Figure 4). Considering that CBS is mainly distributed in mitochondria,[35] we speculate that H_2S produced in mitochondria by CBS can diffuse to the nucleus to trigger cellular signaling. Hydrogen peroxide (H_2O_2) and NO are known for their fast metabolism and short diffusion range. Based on our observation, different from NO and H_2O_2, H_2S may act as a long-range signaling molecule.

In summary, the protein labeling technology provides an opportunity to direct the intracellular distribution of H_2S probes. We developed coumarin based H_2S probes, which can be specifically localized to nucleus and mitochondria depending on the tag fusion proteins used. This strategy can be extended to other fluorophores and cell labeling techniques, such as HaloTag®, and applied to other organelles and H_2S producing enzymes, such as CBS and CSE, to elucidate H_2S related biology.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

This work was supported in part by the National Institutes of Health (R01-GM115622 to J.W.; R01-AG045183 to M.C.W.), the Welch Foundation (Q-1798 to J.W.), and Cancer Prevention and Research Institute of Texas (CPRIT R1104 to J.W.), Centre for Drug Discovery, the Integrated Microscopy Core, the Optical Imaging and Vital Microscopy Core, the Cytometry and Cell Sorting Core, the Dan L. Duncan Cancer Centre at Baylor College of Medicine and the Texas Medical Centre Digestive Diseases Centre (NIDDK 56338).

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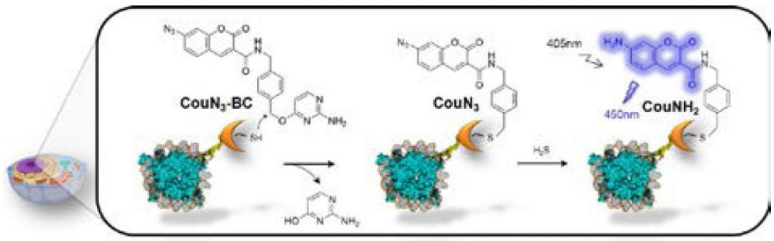

Figure 1.

Illustration of genetically anchored fluorescent probes for subcellular specific imaging of hydrogen sulfide. In this specific example, cyan represents a nucleus localizing protein; orange represents CLIP-tag enzyme, which can specifically react with a CLIP-tag substrate $CouN_3$-BC. Upon reacting with H_2S, non-fluorescent $CouN_3$ is converted to fluorescent $CouNH_2$.

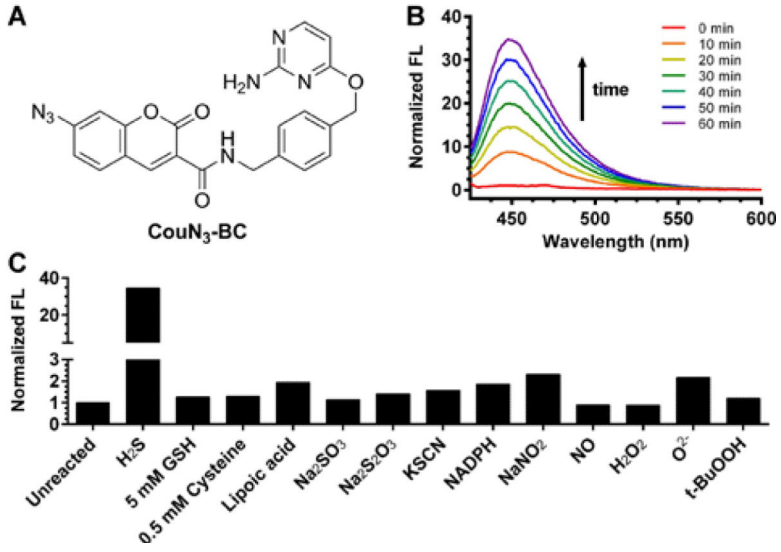

Figure 2.

Reaction specificities of H_2S fluorescent probe $CouN_3$-BC. (A) Chemical structure of organelle specific H_2S probe $CouN_3$-BC. (B) Normalized time dependent fluorescence emission spectra of $CouN_3$-BC (10 μM, λ_{ex} = 405 nm) upon reacting with 100 μM of H_2S in PBS (pH 7.4). The spectra were normalized to the emission intensity at 450 nm at time 0. (C) Normalized fluorescence changes of $CouN_3$-BC (10 μM, λ_{ex} = 405 nm, λ_{em} = 450 nm) upon reacting with a series of redox species in PBS (pH 7.4) for 1 h. The concentrations of GSH and cysteine are 5 mM and 500 μM, respectively. The concentrations of all the other redox species, including H_2S, are 100 μM. The fluorescence intensities at 450 nm were normalized to that of unreacted $CouN_3$-BC. *Note:* All the reactions were carried out under dark and anaerobic conditions to avoid potential photodegradation of the probe and oxidation of reductive species.

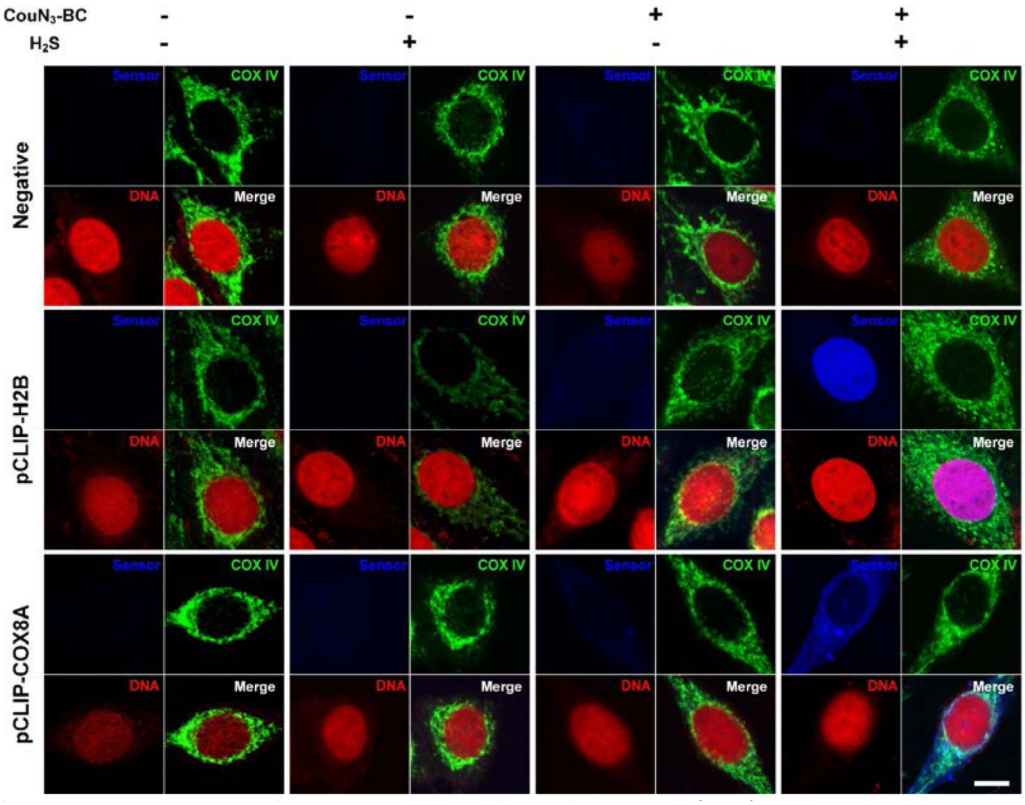

Figure 3.

Confocal microscopic study of the organelle specificity of $CouN_3$-BC (blue) and its responsiveness to cellular H_2S concentration changes. HeLa cells expressing pCLIP-H2B, pCLIP-COX8A, or a control plasmid were incubated with $CouN_3$-BC (10 μM), or NaHS (250 μM) or both. The organelle specificity was confirmed by a secondary staining with either NucRed® Live 647 nuclear stain (red) or an Alexa 488 conjugated antibody against COX-IV (green). Quantitative colocalization analyses showed a significant correlation based on the Pearson's and Manders' coefficients (Table S1). Scale bar is equal to 10 μm.

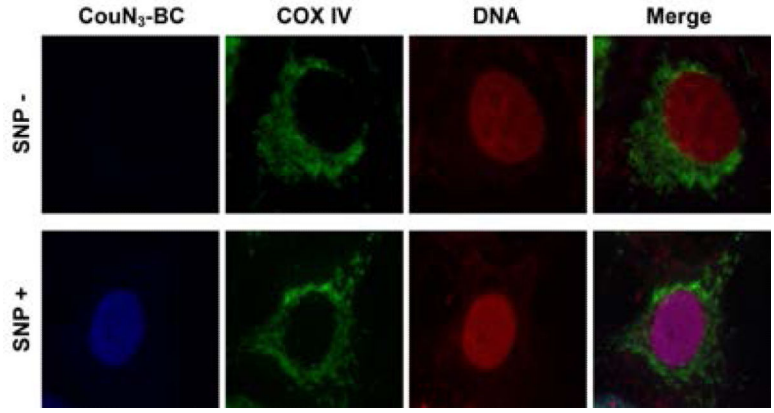

Figure 4.

Confocal microscopic study of the nuclear specific $CouN_3$-BC (blue) and its responsiveness to H_2S concentration changes induced by SNP, an NO donor. HeLa cells expressing pCLIP-H2B were incubated with $CouN_3$-BC (10 μM), and then treated with or without SNP (100 μM). The organelle specificity was confirmed by a secondary staining with either NucRed® Live 647 nuclear stain (red) or an Alexa 488 conjugated antibody against COX-IV