Gasotransmitter Regulation of Phosphatase Activity in Live Cells Studied by Three-Channel Imaging Correlation

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Gasotransmitter Regulation of Phosphatase Activity in Live Cells Studied by Three-Channel Imaging Correlation

Pan Ou, Ruilong Zhang,* Zhengjie Liu, Xiaohe Tian, Guangmei Han, Bianhua Liu, Zhangjun Hu, and Zhongping Zhang*

Abstract: Enzyme activity in live cells is dynamically regulated by small molecular transmitters for maintaining normal physiological functions. A few probes have been devised to measure intracellular enzyme activities by fluorescent imaging, but the study of the regulation of enzyme activity via gasotransmitters *in situ* remains a long-standing challenge. Herein, we report a three-channel imaging correlation by a single dual-reactive fluorescent probe to measure the dependence of phosphatase activity on the H_2S level in cells. The two sites of the probe reactive to H_2S and phosphatase individually produce blue and green fluorescent responses, respectively, and resonance energy transfer can be triggered by their coexistence. Fluorescent analysis based on the three-channel imaging correlation shows that cells have an ideal level of H_2S to promote phosphatase activity up to its maximum. Significantly, a slight deviation from this H_2S level leads to a sharp decrease of phosphatase activity. The discovery further strengthens our understanding of the importance of H_2S in cellular signaling and in various human diseases.

Enzymes are the most critical life substances that catalyze almost all biological reactions such as protein synthesis, energy production and exploitation, and gene expression in cells.^[1,2] Thus, the level and activity of enzymes significantly affect the physiological functions of live organisms, and are highly related to various human diseases.^[2] While working only at suitable temperatures and pH environments, many enzymes are temporally activated or inhibited when they are needed in live organisms.^[3] In molecular biology it is well known that enzyme activity is intrinsically controlled through one or more pathways of signaling transduction, mainly including the regulation of small molecules and the post-

modification of enzymes themselves.^[2] The two pathways determining the activity of an enzyme commonly coexist in live cells. Unlike the study of enzyme activity *in vitro*, multiple correlations seriously increase difficulties in understanding the regulatory and catalytic mechanisms of enzymes.

Among a myriad of enzymes, phosphatase is an important member of the hydrolase family that dephosphorylate proteins or other enzymes and modulate their biological functions. The activity of phosphatase itself is controlled by other upstream signaling molecules such as reactive oxygen species (ROS) and gasotransmitters.^[3] Of these signaling molecules, hydrogen sulfide (H_2S) as a crucial regulator has been studied extensively to clarify its actions on how it affects phosphatase activity, such as its reaction with cysteine (Cys) residue, its coordination with metal ions, and the elimination of ROS.^[4] Meanwhile, phosphatase activity is also indirectly modulated by H_2S through the reaction with phosphatase upstream biomolecules.^[5] Recently, phosphatase activity and the H_2S level in live cells have individually been studied by numerous scientists. Due to their high sensitivity and selectivity, fluorescent probes provide a feasible approach for measuring phosphatase activity; a phosphate-conjugated fluorophore serves as a substrate, and cleavage of phosphate by phosphatase leads to a fluorescent “turn on”.^[6,7] Meanwhile, fluorescent probes for H_2S imaging have also been designed by a series of strategies such as quenching-ion removal,^[8] substitution/addition reactions,^[9] and azido ($-N_3$) group reduction.^[9a,10,11] Although numerous individual phosphatase and H_2S probes are available, evaluating the correlation of phosphatase activity to the H_2S level has been limited because the synchronous use of two fluorescent probes inevitably leads to large invasive effects, different cellular uptake, and spectral interruption.^[12,13]

To bypass these barriers, an ideal strategy is to develop a single fluorescent probe with two different reactive sites that can respond to both phosphatase and H_2S simultaneously with the different spectral signals. Herein, we report a simple but effective method by conjugating H_2S -sensitive and phosphatase-sensitive fluorophores in a single molecular probe to simultaneously measure the H_2S level and the phosphatase activity in live cells. The blue (to H_2S) and green (to phosphatase) fluorescent responses together with their resonance energy transferring signal provide the three-channel fluorescent imaging of H_2S level, phosphatase activity, and their correlation. The current work reveals for the first time that the slight deviation of the H_2S level from an ideal value leads to the sharp decrease of phosphatase activity in live cells.

[*] P. Ou, R. Zhang, Z. Liu, X. Tian, Prof. Z. Zhang
School of Chemistry and Chemical Engineering
and Institute of Physical Science and Information Technology
Anhui University
Hefei, Anhui 230601 (China)
E-mail: zrl@ahu.edu.cn
zpzhang@iim.ac.cn

G. Han, B. Liu, Prof. Z. Zhang
Institute of Intelligent Machines, Chinese Academy of Sciences
Hefei, Anhui 230031 (China)

Z. Hu
Department of Physics, Chemistry and Biology
Linköping University, Linköping 58183 (Sweden)

Supporting information and the ORCID identification number(s) for the author(s) of this article can be found under:
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Here, we selected coumarin (emission at 445 nm) and rhodol (emission at 545 nm) to construct the molecular probe, following two main considerations: 1) the two emissive peaks were separated by roughly 100 nm to avoid spectral interference; 2) the emission spectra of coumarin overlaps with the absorption of rhodol (Figure S1), leading to Förster resonance energy transfer (FRET).^[14] Figure 1a gives the probe structure that comprises a two fluorophores, N₃-modified coumarin and phosphate-modified rhodol, connected by a piperazine bridge. The synthetic protocol is shown in Scheme S1 of the Supporting Information. Simply, a carboxyl coumarin was synthesized and further modified with -N₃, which shut off the blue emission due to the strong electron-withdrawing effect of this functional group.^[9] Then, the coumarin derivative was linked to rhodol with a piperazine

bridge through an amidation reaction. Finally, phosphate was grafted onto rhodol to obtain our probe N₃-CR-PO₄; the green emission of rhodol is shut off since spirocyclization interrupts the π -conjugation.^[13] After purification, N₃-CR-PO₄ was characterized by HRMS, and ¹H, ¹³C and ³¹P NMR spectroscopy (Figures S2–S5).

On the one hand, the blue fluorescence of probe N₃-CR-PO₄ is turned on by H₂S via the reduction of -N₃ to -NH₂; on the other hand, its phosphate group can be catalytically cleaved by phosphatase, leading to recovery of the green fluorescence of rhodol (Figure 1a). In the presence of both H₂S and phosphatase, only green fluorescence is detected with the optimal excitation wavelength of coumarin, owing to FRET from coumarin to rhodol. However, this signal is different from the green emission induced by phosphatase alone, which is rather weak at this excitation wavelength. The reaction of the probe with H₂S and phosphatase has been certified by high-performance liquid chromatography (Figure S6).

The sensitivity of the probe was examined by a series of measurements of fluorescent responses to H₂S and alkaline phosphatase (ALP) in Tris-HCl buffer (Figure 1 b–d). Before the test, the original probe showed two weak fluorescence peaks at 445 and 545 nm (excitation: 360 nm; red curve in Figure 1 b). Upon addition of H₂S to the N₃-CR-PO₄ solution, the fluorescent peaks at 445 nm gradually became stronger, and finally reached ≈ 13 times enhancement at 70 μ M H₂S. Even at 1 nM H₂S, ≈ 1.4 -fold fluorescence enhancement indicated the high sensitivity of the probe to H₂S. The fluorescence intensity exhibited a linear relationship vs. H₂S amount from 10 to 60 μ M (standard deviation $R^2 = 0.999$, Figure S8). Meanwhile, the blue fluorescence could be clearly seen under a UV lamp (inset in Figure 1 b). It was noted that the other weak emission at 545 nm remained almost unchanged throughout the titration.

With the addition of ALP, the probe as a substrate of phosphatase showed an apparent and continuous increase of fluorescent peak at 545 nm (excitation: 510 nm; Figure 1 c). The maximum enhancement was up to ≈ 23 fold at 2 U L⁻¹ ALP. Even at 0.01 U L⁻¹ ALP, fluorescence still increased by a factor of 2, suggesting the supersensitivity of the probe to phosphatase. We observed that the solution color changed from colorless to bright green under a UV lamp (inset in Figure 1 c). The fluorescence intensities exhibited a linear relationship against ALP amounts from 0.01 to 1 U L⁻¹ (standard deviation $R^2 = 0.995$, Figure S9).

Because the coexistence of H₂S and ALP is very common in biological systems, the performance of the probe in this situation was verified in vitro by the addition of both H₂S and ALP. A 10 μ M solution of the probe was first mixed with 50 μ M H₂S; this is close to the average H₂S level in many biological samples.^[15] After 20 min the solution showed a blue emission at 445 nm (excitation: 360 nm). With the further addition of ALP to this solution, the peak at 445 nm decreased rapidly, while the fluorescence at 545 nm remarkably increased up to ≈ 7 -fold (Figure 1 d). These phenomena confirm the occurrence of efficient FRET from coumarin to rhodol (Figure 1 a). In the absence of H₂S, however, only a slight enhancement of green fluorescence was detected upon excitation at 360 nm

Figure 1. a) Fluorescent response mechanisms of probe N₃-CR-PO₄ to H₂S, phosphatase, and H₂S/phosphatase mixture. b) Fluorescence spectra of 10 μ M N₃-CR-PO₄ with the addition of H₂S in Tris-HCl buffer (pH 7.2) for 1 h; excitation: 360 nm. c) Fluorescence spectra of 10 μ M N₃-CR-PO₄ with the addition of ALP in Tris-HCl buffer (pH 8.0); excitation: 510 nm; the insets are the corresponding photographs under 365 nm UV lamp. d) Fluorescence spectra upon mixing 10 μ M N₃-CR-PO₄ and 50 μ M H₂S and adding ALP; excitation: 360 nm (pH 8.0). e) The selectivity of fluorescent responses (I/I_0) to various biomolecules in 10 μ M N₃-CR-PO₄ solution. 1: blank, 2: 60 μ M H₂S, 3: 2 U L⁻¹ ALP, 4: 100 mg L⁻¹ ACP, 5: 60 μ M Na₂S₂, 6: 60 μ M Na₂S₂O₆, 7: 100 μ M NaSCN, 8: 100 μ M Na₂SO₃, 9: 100 μ M Na₂S₂O₃, 10: 100 μ M Cys, 11: 100 μ M GSH, 12: 10 U L⁻¹ AChE, 13: 10 U L⁻¹ GDH, 14: 10 U L⁻¹ GOX, 15: 10 U L⁻¹ thrombin, 16: 250 mg L⁻¹ trypsin, 17: 10 U L⁻¹ PDE. The error bars represent the mean errors from the results of 5 tests.

(Figure S10). In comparison, $\approx 72\%$ fluorescence enhancement at 545 nm results from FRET in the coexisting system. The spectral signal forms the basis of the third-channel detection in fluorescent imaging (see the following text).

Before transferring the *in vitro* experiments to cellular imaging, we tested the effects of pH on the fluorescent signals of the probe sensing H_2S and phosphatase. In the range of pH 5–9, the probe itself remained almost non-fluorescent; however, its three products formed by reaction with H_2S , phosphatase, and both of them (Figure 1a) exhibited strong and stable fluorescence (Figure S11). Similar to the previously reported probe,^[16] the protonation of the piperazine group slightly decreased the green fluorescent signal from phosphatase at pH 5.

Moreover, we examined the selectivity of the probe using several kinds of molecules that commonly exist in live cells, including amino acids, peptides, proteins, enzymes, and inorganic salts (Figure 1e). The additions of Cys, glutathione (GSH), glucose oxidase (GOX), acetylcholinesterase (AChE), phosphodiesterase (PDE), glucose dehydrogenase (GDH), thrombin, trypsin, Na_2S_2 , $\text{Na}_2\text{S}_{2-6}$, NaSCN , Na_2SO_3 , and $\text{Na}_2\text{S}_2\text{O}_3$ to the probe solution did not cause any significant fluorescence enhancement upon excitation at either 360 or 510 nm. Only H_2S induced strong blue emission (excitation: 360 nm), while phosphatase including ALP and acid phosphatase (ACP) remarkably enhanced green emission at 545 nm. Particularly, polysulfides still did not trigger the fluorescent response of probe, suggesting the high selectivity of probe to H_2S and phosphatase.

Before fluorescent imaging, the toxicity of probe was evaluated by classical MTT assay (Figure S12), and the results showed that our probe was nontoxic to cells in the concentration range of 1–20 μM .

Next, we set a three-channel imaging procedure to simultaneously detect H_2S and phosphatase: CH1: excitation at 405 nm and collection in 420–470 nm for H_2S ; CH2: excitation at 510 nm and collection in 530–570 nm for phosphatase; CH3: excitation at 405 nm and collection in 530–570 nm for FRET correlation for coexisting H_2S and phosphatase (Table S1). When HeLa cells were treated with 10 μM probe alone, all of the channels gave strong fluorescent signals (Figure 2a), indicating that endogenous H_2S and phosphatase are sufficient to turn on the fluorescence of the probe in live cells. Especially, the bright fluorescence in CH3 suggests a highly efficient reaction of probe with both H_2S and phosphatase. To confirm the origin of the fluorescence signals, negative control experiments were performed by employing a phosphatase inhibitor (Na_3VO_4)^[6a] and an H_2S -generating enzyme inhibitor (aminooxyacetic acid, AOAA).^[17] After the cells were sequentially treated with Na_3VO_4 and the probe, the fluorescences in CH2 and CH3 from phosphatase obviously reduced, but the fluorescent signal in CH1 from H_2S became slightly stronger due to the interruption of FRET when phosphatase activity had been inhibited greatly (middle column of Figure 2a). Indeed, green fluorescence results from the phosphatase and FRET between the two fluorophores in cells.

In another control group, the addition of AOAA would inhibit the further production of endogenous H_2S to reduce

Figure 2. Simultaneous visualization of H_2S level and phosphatase activity in HeLa cells. a) Left column: HeLa cells were treated with 10 μM $\text{N}_3\text{-CR-PO}_4$ for 30 min; Middle column: HeLa cells were treated with 100 μM Na_3VO_4 for 30 min and then incubated with 10 μM $\text{N}_3\text{-CR-PO}_4$; Right column: HeLa cells were treated with 100 μM AOAA for 30 min, and then incubated with 10 μM $\text{N}_3\text{-CR-PO}_4$. b) Static fluorescence intensities of (a). The error bars represent standard deviation ($\pm$ SD).

the level of H_2S in cells, leading to a weaker fluorescence in CH1 (right column of Figure 2a). Surprisingly, the fluorescent signals from CH2 and CH3 became rather weak, roughly commensurate with those upon the addition of phosphatase inhibitor Na_3VO_4 (Figure 2b). This suggests that the decrease of the H_2S level may result in phosphatase inactivation in live cells.

To verify H_2S regulation of phosphatase activity, we treated cells with the H_2S scavenger phorbol 12-myristate 13-acetate (PMA) to decrease intracellular H_2S level, and then subsequently added 10 μM probe solution. With increasing the PMA dose, the fluorescent signals from all three channels became gradually weaker (Figure 3a,b). Particularly, the signals in CH2 and CH3 almost disappeared. That is, the decrease in the H_2S level greatly inhibits the activity of phosphatase, confirming H_2S as an activator to maintain the normal activity of phosphatase in cells.

To further examine the effect of H_2S on phosphatase activity, we tried to study the performance of phosphatase at elevated H_2S levels in cells. HeLa cells were treated with different doses of H_2S and then incubated with 10 μM probe solution. As shown in Figure 3c and 3d, the fluorescent signal from CH1 was gradually enhanced with the increase of H_2S . In contrast, however, the signals in CH2 and CH3 became weaker and weaker. With the addition of only 10 μM H_2S , the signal intensity in CH2 decreased at least to half. More H_2S supplement (e.g. 60 μM) led to almost disappearance of CH2 signal (Figure 3d). The same results were also obtained in other cell lines (Figures S13 and S14). Together with these above results, it is evident that phosphatase activity rapidly decreases when the concentration of H_2S deviating from its normal level in cells.

It should be noted that the blue and green fluorescences did not completely overlap in the three-channel imaging. The inconsistent localizations should result from the different products produced by the probe reacting with H_2S and phosphatase. To clarify this, we have performed the co-

Figure 3. The dependence of phosphatase activity on H₂S level in live cells. a) HeLa cells were treated with different concentrations of H₂S scavenger (PMA) for 3 h and then incubated with 10 μM N₃-CR-PO₄. b) Statics fluorescence intensities corresponding to the fluorescent imaging in (a). c) HeLa cells were treated with different concentrations of H₂S for 30 min and then incubated with 10 μM N₃-CR-PO₄. d) Statics fluorescence intensity corresponding to the fluorescent imaging in (c). The error bars represent standard deviation ($\pm$ SD).

localization experiments of the three different products (obtained from the pre-reaction in vitro as in Figure 1a). Indeed, most of the products from the reactions of the probe with H₂S and H₂S/phosphatase entered the lysosomes in cells by co-localization, while the reaction product of the probe with phosphatase alone randomly distributed in cytoplasm (Figure S15).

Moreover, we have also performed control experiments by combining two commercial probes: 7-azidocoumarin for H₂S and fluorescein diphosphate for phosphatase. The blue and green fluorescent signals for H₂S and phosphatase, respectively, exhibited the same trends displayed in experiments with our single probe (Figure 3 and Figure S16). Here, a detected difference from our results is that the addition of H₂S scavenger sharply decreased the activity of phosphatase almost to its minimum. One main reason may be the high cytotoxicity from the combination of the two probes (Figure S12); the cell viability decreased by 60% at the probe concentration used (10 μM). The toxic stress would stimulate cells to generate more ROS which could further decrease the H₂S level in the cells, greatly lowering phosphatase activity.

We have also used H₂S and phosphatase kits to verify the above results. First, HeLa cells were incubated with PMA and H₂S to lower and elevate intracellular H₂S levels, respectively. After washing, these cells were lysed to obtain cell lysates, and the H₂S level and phosphatase activity were measured by kits. Figure 4 shows that the phosphatase activity is the highest in the control group with the normal level of H₂S ($\approx$ 55 μM). When the H₂S level rose or fell by $\approx$ 10 μM, phosphatase

activity sharply decreased by at least 50%, and then showed a continuous decrease with further deviations of H₂S level from the normal value, which was in agreement with the results from fluorescent imaging.

Previous biological studies mainly focused on phosphatase activity at elevated H₂S levels,^[4d,e] and showed the inhibition of phosphatase activity with the increase of intra-

Figure 4. Relation between H₂S level and phosphatase activity. The H₂S level and phosphatase activity were measured in cell lysates by kit tests. In the control groups, HeLa cells were untreated. In the group treated with PMA, the cells were incubated with different amounts of PMA for 3 h. In group treated with H₂S, the cells were incubated with different concentrations of H₂S for 30 min. The error bars represent the mean errors from the results of 3 tests.

cellular H₂S level, which is consistent with our results here. However, phosphatase activity with a decrease of intracellular H₂S level has been rarely explored. In our present work we found that there is an critical H₂S level in cells to control the activity of phosphatase.

In summary, we have successfully designed and synthesized a dual-reactive molecular probe that can respond to both H₂S and phosphatase with two differentiable fluorescent signals. The combination with the FRET signal between these fluorescence signals established a three-channel imaging method to detect H₂S and phosphatase activity in live cells, and to clarify the correlation of phosphatase activity with H₂S level. Importantly, the results reported here reveal that cells have an ideal H₂S level to maintain the high activity of phosphatase, and the slight deviations from the ideal value will significantly reduce the activity of phosphatase. This significant discovery indicates that fluorescent probes will become a powerful tool to explore the regulatory mechanisms of enzyme activity in live organisms.

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Conflict of interest

The authors declare no conflict of interest.

Keywords: enzyme regulation · fluorescent probes · gasotransmitters · imaging agents · phosphatase

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Communications

Fluorescent Probes

P. Ou, R. Zhang,* Z. Liu, X. Tian, G. Han,
B. Liu, Z. Hu, Z. Zhang* — ■■■—■■■

Gasotransmitter Regulation of
Phosphatase Activity in Live Cells Studied
by Three-Channel Imaging Correlation

Two for one: A dual-reactive molecular probe has been devised to simultaneously measure intracellular H_2S levels and phosphatase activity. Fluorescent analysis by three-channel imaging correlation reveals that a slight deviation from the normal H_2S level leads to a sharp decrease of phosphatase activity.

Fluoreszenzsonden

P. Ou, R. Zhang,* Z. Liu, X. Tian, G. Han,
B. Liu, Z. Hu, Z. Zhang* — ■■■—■■■

Gasotransmitter Regulation of
Phosphatase Activity in Live Cells Studied
by Three-Channel Imaging Correlation

Eine molekulare Sonde mit dualer Reaktivität kann simultan intrazelluläre H_2S -Niveaus und Phosphatase-Aktivität messen. Eine Fluoreszenzanalyse durch Drei-Kanal-Imaging-Korrelation ergibt, dass eine leichte Abweichung vom normalen H_2S -Niveau zu einer drastischen Abnahme der Phosphatase-Aktivität führt.

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Pan Ou

Ruilong Zhang

Zhengjie Liu

Xiaohe Tian

Guangmei Han

Bianhua Liu

Zhangjun Hu

Prof. Zhongping Zhang <http://orcid.org/0000-0002-0991-7611>