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Letter

Chemogenetic Manipulation of H_2S with Spatiotemporal Precision

Asal Ghaffari Zaki, Hamzah Issa, Seyed Mohammad Miri, Joudi Armouch, Asel Aydeger, Sena Yildirim, Refia Zeynep Mete, Omar Aljundi, Emre Vatandaşlar, Tuba Akgul Caglar, Şeyma Çimen, Esra Nur Yiğit, Mehmet Şerif Aydın, Muhammed İkbal Alp, Toghrul Almammadov, Sven Vilain, and Emrah Eroglu*

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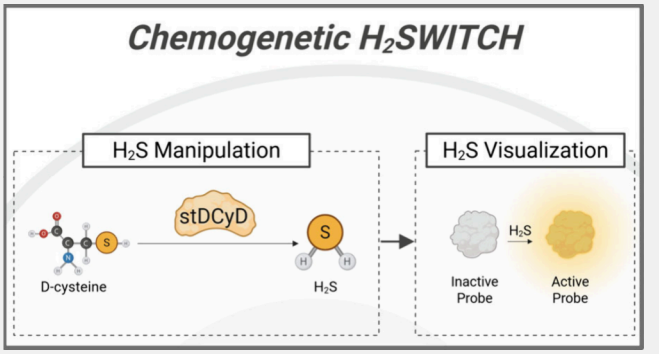
ABSTRACT: Hydrogen sulfide (H_2S) is a signaling molecule with a plethora of biological functions, yet precision tools for modulating its intracellular flux remain scarce. Conventional small-molecule donors and enzymatic systems often suffer from off-target reactivity, uncontrolled release kinetics, and redox crosstalk, confounding mechanistic studies. Here, we establish a *Salmonella typhimurium* D-cysteine desulfhydrase (stDCyD)-derived chemogenetic tool for controlled H_2S manipulation in living cells. stDCyD catalyzes the α,β-elimination of D-cysteine to selectively yield bioavailable H_2S. We term this tool H_2SWITCH. Our approach exhibits pronounced enantioselectivity for D-cysteine, robust catalytic efficiency at physiological temperatures, and temporal tunability through substrate dosing. This chemogenetic tool provides a chemically defined and interference-free method to unravel the physiological and pathological roles of H_2S with unprecedented precision in complex biological systems.



KEYWORDS: *Hydrogen sulfide, D-cysteine, Chemogenetics, Redox signaling, Salmonella typhimurium D-cysteine desulfhydrase*

Hydrogen sulfide (H_2S) has emerged as a critical gasotransmitter alongside nitric oxide (NO) and carbon monoxide (CO), with growing recognition of its diverse roles in physiological and pathological processes.[1,2] Originally regarded as merely a toxic gas, H_2S is now known to be endogenously produced in mammalian cells by enzymes such as cystathionine β-synthase (CBS), cystathionine γ-lyase (CSE), and 3-mercaptopyruvate sulfurtransferase (MPST).[3−5] H_2S regulates key biological functions including vascular tone,[6] mitochondrial bioenergetics[7,8] and neuromodulation.[9] Its homeostatic imbalance has been implicated in a range of disorders.[10−12]

Traditional approaches to modulate intracellular H_2S levels largely rely on genetic manipulations, such as the overexpression or knockout of H_2S-producing enzymes,[13] or pharmacological interventions, including the administration of sulfide-releasing salts like sodium hydrosulfide (NaHS) or slow-releasing H_2S donors.[14] However, these methods suffer from major drawbacks, including poor spatiotemporal resolution, a lack of reversibility, and off-target effects that complicate the interpretation of results. Particularly in single-cell studies or in complex tissue environments, the inability to achieve precise, real-time control of H_2S concentrations limits the scope of mechanistic investigations. In recent years, several chemical probes, such as Reso-N3,[15] resorufin-functionalized coumarin probe (RC),[16] and resorufin-based probe RH[17] for detection of H_2S and thiols have been developed. Besides, genetically encoded sensors for real-time detection of H_2S have been established (hsGFP[18] and hsFRET[19] biosensors). These advances have provided valuable insights into the spatial and temporal dynamics of H_2S signaling. However, detection alone is insufficient for functional studies; tools are needed that not only report on H_2S levels but also enable active manipulation with high precision.

In our previous work, we demonstrated that modified D-amino acid oxidase (mDAAO) can catalyze H_2S production in the presence of D-cysteine.[20] In a recent study, we introduced a chemogenetic approach for manipulation of intracellular pH, named pH-Control,[21] using the *Salmonella typhimurium* D-cysteine desulfhydrase (stDCyD) enzyme. In the presence of its substrate β-chloro-D-alanine (βCDA), stDCyD generates significant levels of hydrochloric acid (HCl) along with the byproducts, pyruvate, and ammonia in negligible amounts. In this study, we establish and characterize stDCyD as a chemogenetic tool that, in the presence of D-cysteine, can generate H_2S in living mammalian cells. By introducing a robust, targeted, and highly specific strategy to control intracellular H_2S

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concentrations, we provide a powerful tool to unravel the complex biology of this important gaseous signaling molecule.

As shown in Figure 1, stDCyD directly converts D-cysteine into H_2S, pyruvate and ammonium (NH_4^+), utilizing pyridoxal

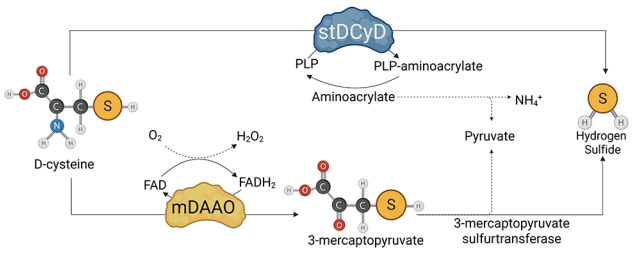

Figure 1. Mechanism of H_2S production by stDCyD and mDAAO. The stDCyD enzyme catalyzes the conversion of D-cysteine to H_2S, with the coenzyme pyridoxal 5′-phosphate (PLP) forming an aminoacrylate intermediate. This intermediate is subsequently converted to pyruvate and ammonium. In contrast, mDAAO oxidizes D-cysteine to 3-mercaptopyruvate using flavin adenine dinucleotide (FAD) as a cofactor, producing H_2O_2 as a byproduct. The resulting 3-mercaptopyruvate is then converted to H_2S by the endogenous enzyme 3-mercaptopyruvate sulfurtransferase, generating pyruvate as a by-product.

5′-phosphate (PLP) as a cofactor. In contrast, mDAAO first oxidizes D-cysteine into its corresponding α-keto acid, mercaptopyruvate, which is subsequently metabolized to H_2S by endogenous MPST. Both MPST and stDCyD produce pyruvate as a byproduct. Therefore, to indirectly compare the efficiencies of these two enzymatic pathways (as well as the endogenous pathway (MPST)), we used a genetically encoded pyruvate biosensor to monitor this metabolite's production in real time[22] (Figure S1). Our results showed that significant pyruvate generation occurred only in cells expressing stDCyD, but not in wild-type (WT) cells or those expressing mDAAO (Figure S1). These findings suggest that stDCyD exhibits superior catalytic efficiency for D-cysteine metabolism under the tested conditions.

Further characterization of the enzymatic activities focused on H_2O_2 generation. It is well established that mDAAO, while catalyzing the oxidation of D-amino acids, produces H_2O_2 as a byproduct. Live-cell H_2O_2 imaging revealed that, in mDAAO-expressing cells, 5 mM D-cysteine produced intracellular H_2O_2 levels comparable to those induced by 50 μM exogenous H_2O_2 (Figure S2). In contrast, cells expressing stDCyD showed no detectable H_2O_2 production under the same conditions (Figure S2). This distinction further highlighted the advantage of stDCyD for specific H_2S manipulation without the confounding effects of the oxidative stress induced by H_2O_2. Based on these observations, we selected stDCyD as the preferred chemogenetic tool for further characterization.

In a cell-free ("*in chemico*") assay, we quantified H_2S production by purified stDCyD using RH probe.[17] (See Supplementary Note 1 for details.) As shown in Figure 2B, purified stDCyD was incubated with D-cysteine and H_2S formation was monitored in real time via RH fluorescence. In control reactions lacking stDCyD, increasing D-cysteine produced only a slight fluorescence rise, consistent with the probe's low background reactivity toward the thiol of D-cysteine (Figure 2C). In the presence of stDCyD, RH fluorescence increased in a D-cysteine concentration-dependent manner and exceeded no-enzyme controls, especially at higher D-cysteine

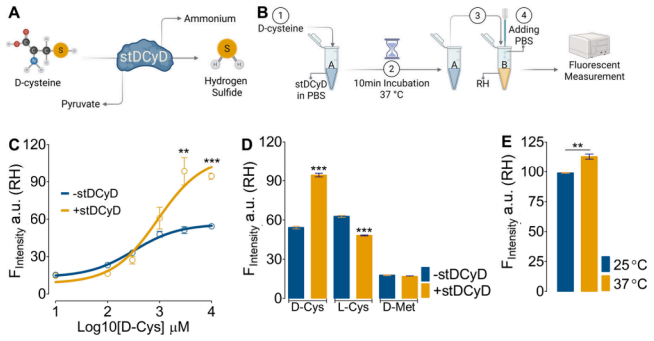

Figure 2. Characterization of H_2SWITCH using the RH probe. A) Schematic representation of the RH probe and its activation by H_2S. B) Schematic illustration of the experimental protocol for H_2S measurement based on the enzymatic activity of stDCyD using the RH fluorescent probe. Desired concentrations of D-cysteine were mixed with purified stDCyD in PBS and incubated for 10 min at 37 °C. Subsequently, 2 μL of the reaction mixture was added to the RH probe solution, followed by PBS to reach the final assay volume for fluorescence measurements using a plate reader. C) Dose-dependent generation of H_2S by stDCyD in the presence of indicated concentrations of D-cysteine (orange curve). The blue curve represents the response of the RH probe to D-cysteine alone. D) RH fluorescence changes in response to 10 mM D-cysteine, 10 mM L-cysteine, and 10 mM D-methionine in the presence or absence of stDCyD. E) Quantification of H_2S generation by stDCyD at room temperature (25 °C) and physiological temperature (37 °C). Statistical analysis for C, D, E: $n = 3$ for all experiments, and two-tailed Student's t test was applied to compare two independent groups; *** $P < 0.001$. D-Cys: D-cysteine. L-Cys: L-cysteine. D-Met: D-methionine.

concentration (Figure 2C). To assess substrate specificity, we compared RH responses to D-cysteine, L-cysteine, and D-methionine with or without stDCyD (Figure 2D). D-cysteine plus stDCyD elicited a strong signal, consistent with efficient H_2S formation. L-cysteine decreased fluorescence relative to the enzyme-free control, consistent with competitive binding to stDCyD without turnover and suppression of the residual probe background (Figure 2D). D-Methionine had no measurable effect on the RH response (Figure 2D). Fluorescence was higher at 37 °C than at 25 °C (Figure 2E), indicating greater catalytic activity at physiological temperature. In addition, we have investigated the functionality of stDCyD from acidic pH to physiological range (Figure S3) against one of its known substrates, βCDA, and showed that, at neutral pH, stDCyD shows a better catalytic activity (Figure S3). These results validate stDCyD as a selective, physiologically compatible, and dose-tunable chemogenetic approach for on-demand generation of H_2S from D-cysteine.

To evaluate the applicability of H_2SWITCH in mammalian cells, we first assessed the potential toxicity of D-cysteine in wild-type HEK 293T cells and in cells stably expressing DsRed−stDCyD−NES (Figure S4). No cytotoxic effects were observed in either cell line at concentrations of up to 30 mM D-cysteine. We then proceeded to generate and directly visualize H_2S *in cellulo*. Wild type HEK 293T cells and cells expressing stDCyD were transiently transfected with the hsGFP[18] sensor (Figure 3A). Administration of 10 mM D-cysteine increased H_2S in both WT and stDCyD-expressing cells (Figure 3B), but the maximal response was significantly greater in the latter (Figure 3C).In our previous study, we demonstrated that the DsRed-stDCyD construct can be selectively targeted to distinct subcellular compartments, where it retained full enzymatic functionality.[21]

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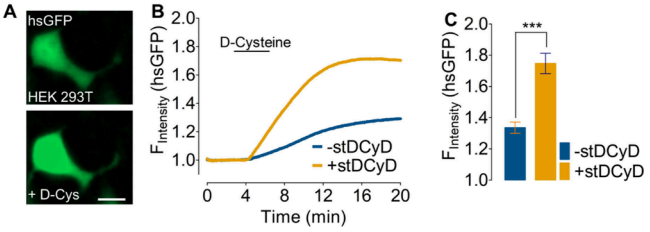

Figure 3. H_2S generation in mammalian cells upon the enzymatic activity of stDCyD. A) Wide-field images show the change in the hsGFP fluorescence upon administration of D-cysteine in cells coexpressing hsGFP and stDCyD enzyme. Scale bar represents 10 μm. B) Panel shows representative real-time traces of produced H_2S in response to 10 mM D-cysteine in HEK 293T cells coexpressing untargeted hsGFP and stDCyD (orange curve, 3/41) or only hsGFP (blue curve, 3/54). C) The bar graph shows statistical analysis of the maximum response of hsGFP in response to 10 mM D-cysteine. Statistical analysis was performed using Student's *t* test. *** refers to $P < 0.001$.

To further verify compartment-specific H_2S generation, we employed a mitochondria targeted DsRed-stDCyD together with an untargeted hsGFP biosensor (Figure S5). Our data show that stDCyD efficiently metabolizes D-cysteine to produce H_2S and validate its utility as a robust tool for intracellular H_2S generation. The modest H_2S signal in WT cells upon D-cysteine treatment can be explained by (i) oxidation of D-cysteine to mercaptopyruvate by endogenous D-amino-acid oxidase followed by MPST-mediated H_2S formation,[23] (ii) racemization of D-cysteine to L-cysteine (e.g., via serine racemase),[24] enabling metabolism by canonical H_2S-producing enzymes (CBS, CSE), and (iii) the basal reactivity of the probe with D-cysteine. Regarding the latter, we showed that *p*AzF-treated HEK 293T cells respond to D-cysteine addition, evidenced by an increase in the GFP-channel fluorescence intensity (Figure S6A). Moreover, a highly significant increase in fluorescence intensity was observed when *p*AzF and D-cysteine were incubated together in a cell-free buffer (Figure S6B). Together, these data underscore the urgent need for more specific probes for H_2S visualization.

In conclusion, we present H_2SWITCH, a substrate-gated chemogenetic strategy that enables dose-tunable, enantioselective, and redox-orthogonal modulation of intracellular H_2S, which establish H_2SWITCH as a precise, interference-free tool for manipulating H_2S flux. Mechanistically, stDCyD offers several advantages: (i) orthogonality—the D-cysteine/stDCyD pair minimizes engagement of native pathways; (ii) spatiotemporal control, substrate dosing affords rapid on-/off-like behavior without slow donor kinetics; (iii) compatibility with sensors, H_2S formation can be read out in real time with genetically encoded or chemical reporters, enabling closed-loop experiments. The genetically encodable nature of H_2SWITCH allows precise spatial and temporal control of H_2S production, a feature that is unattainable with small-molecule donors. In contrast to donor-based systems that rely on passive diffusion and trigger-dependent chemical release,[14,25] stDCyD affords on-demand manipulation of H_2S flux within specific cellular microdomains. Such modularity parallels our previous demonstration of the same enzyme scaffold's functional robustness and targeting flexibility in a chemogenetic pH-control system,[21] where compartment-specific localization did not compromise catalytic efficiency. While recent developments in stimuli-responsive H_2S donors have enhanced the temporal control of chemical donors, these molecules still exhibit intrinsic limitations. Specifically, they display poor cell-type selectivity,[26] unpredictable and burst-type release kinetics,[27] limited intracellular stability,[28] and potential off-target oxidation or cytotoxic byproducts that obscure mechanistic readouts.[14,29] Conversely, the H_2SWITCH system provides a genetically precise, catalytically orthogonal, and imaging-compatible means to modulate H_2S signaling, enabling kinetic and spatial dissection of this gasotransmitter's roles in mitochondria, cytosol, or other subcellular niches.

At the same time, important caveats warrant consideration. D-Cysteine can be racemized or enter endogenous routes[23,24] (e.g., DAAO→MPST), which likely explains the modest WT signals (Figure 3B); these effects can be bounded with catalytically dead stDCyD controls, pathway inhibitors and knockdowns (i.e., MPST or serine racemase K.O. cell lines[24]). Second, pyruvate is a stoichiometric byproduct and may influence metabolism;[30] concurrent pyruvate sensing, short pulses, or compartment targeting help deconvolute these effects.[31] Third, effective use depends on intracellular PLP availability;[32] substrate uptake through transporters[33] and media composition should be validated per model.

Our H_2SWITCH approach opens experimental spaces that were previously hard to access. Compartment-resolved H_2S biology becomes tractable by directing stDCyD to mitochondria, ER, or endothelium-specific microdomains to test local persulfidation events, K_{ATP} channel regulation,[34] or crosstalk with NO/CO signaling.[35] Future studies emphasizing subcellular targeting and local H_2S generation (e.g., mitochondrial matrix, cytosol, ER) will elucidate the biological roles of H_2S microdomains in specific pathways.

Kinetic causality—how fast and how much H_2S is required to trigger specific pathways—can be quantified by pairing H_2SWITCH with real-time reporters (H_2S, redox, Ca^{2+}, ATP)[18,36−38] and time-stamped perturbations.

Looking ahead, *in vivo* translation is feasible with AAV-mediated or transgenic delivery and cell-type−specific promoters or Cre lines, combined with localized D-cysteine administration (e.g., cerebral open fluid microperfusion (cOFM).[39] Coupling H_2SWITCH with omics readouts and proteomic persulfidation mapping will enable systems-level causal links between the H_2S flux and phenotype.

In summary, H_2SWITCH provides a precision chemogenetic route to interrogate H_2S signaling with temporal, spatial, and mechanistic clarity. By minimizing redox crosstalk and enabling compartment-specific, dose-controlled generation, it sets the stage for definitive causal tests of H_2S function across vascular, cancer, and neurobiological contexts—experiments that have remained largely out of reach with existing donor or genetic strategies.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/prechem.5c00189.

Detailed materials and methods and supplementary Note 1; Figures S1−S13 of cellulo experiments for the live-cell imaging of pyruvate and hydrogen peroxide dynamics, as well as in chemico characterization of the RH probe (PDF)

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AUTHOR INFORMATION

Corresponding Author

Emrah Eroglu − *Research Institute for Health Sciences and Technologies (SABITA), Istanbul Medipol University, Istanbul 34810, Türkiye; Molecular Biology, Genetics, and Bioengineering Program, Sabanci University, Istanbul 34956, Türkiye;* orcid.org/0000-0002-9373-0808; Email: emrah.eroglu@medipol.edu.tr

Authors

Asal Ghaffari Zaki − *Research Institute for Health Sciences and Technologies (SABITA) and Graduate School of Health Sciences, Istanbul Medipol University, Istanbul 34810, Türkiye*

Hamzah Issa − *Research Institute for Health Sciences and Technologies (SABITA) and Graduate School of Health Sciences, Istanbul Medipol University, Istanbul 34810, Türkiye*

Seyed Mohammad Miri − *Research Institute for Health Sciences and Technologies (SABITA), Istanbul Medipol University, Istanbul 34810, Türkiye;* orcid.org/0000-0003-2753-3221

Joudi Armouch − *Research Institute for Health Sciences and Technologies (SABITA) and Biomedical Engineering and Bioinformatics, Graduate School of Engineering and Natural Sciences, Istanbul Medipol University, Istanbul 34810, Türkiye;* orcid.org/0009-0008-0106-5830

Asel Aydeger − *Research Institute for Health Sciences and Technologies (SABITA) and Graduate School of Health Sciences, Istanbul Medipol University, Istanbul 34810, Türkiye*

Sena Yildirim − *Research Institute for Health Sciences and Technologies (SABITA) and Graduate School of Health Sciences, Istanbul Medipol University, Istanbul 34810, Türkiye*

Refia Zeynep Mete − *Research Institute for Health Sciences and Technologies (SABITA), Istanbul Medipol University, Istanbul 34810, Türkiye;* orcid.org/0009-0005-4472-4232

Omar Aljundi − *Research Institute for Health Sciences and Technologies (SABITA), Istanbul Medipol University, Istanbul 34810, Türkiye*

Emre Vatandaşlar − *Research Institute for Health Sciences and Technologies (SABITA), Istanbul Medipol University, Istanbul 34810, Türkiye*

Tuba Akgul Caglar − *Research Institute for Health Sciences and Technologies (SABITA), Istanbul Medipol University, Istanbul 34810, Türkiye*

Şeyma Çimen − *Department of Nutrition and Dietetics, Faculty of Health Sciences, Bolu Abant Izzet Baysal University, Bolu 14030, Turkey*

Esra Nur Yiğit − *Research Institute for Health Sciences and Technologies (SABITA) and Department of Physiology, International School of Medicine, Istanbul Medipol University, Istanbul 34810, Türkiye*

Mehmet Şerif Aydın − *Research Institute for Health Sciences and Technologies (SABITA) and Department of Histology, School of Medicine, Istanbul Medipol University, Istanbul 34810, Türkiye*

Muhammed İkbal Alp − *Research Institute for Health Sciences and Technologies (SABITA) and Department of Physiology, School of Medicine, Istanbul Medipol University, Istanbul 34810, Türkiye*

Toghrul Almammadov − *Research Institute for Health Sciences and Technologies (SABITA), Istanbul Medipol University, Istanbul 34810, Türkiye*

Sven Vilain − *Research Institute for Health Sciences and Technologies (SABITA) and Department of Medicinal Biology, International School of Medicine, Istanbul Medipol University, Istanbul 34810, Türkiye;* orcid.org/0000-0002-9166-2792

Complete contact information is available at: https://pubs.acs.org/10.1021/prechem.5c00189

Author Contributions

A.G.Z. and E.E. conceived the research idea and designed the experiments. A.G.Z., R.Z.M., and O.A. performed the *in cellulo* experiments. H.I. and S.M.M. carried out the *in chemico* experiments. Ş.Ç. and A.A. designed and performed Seahorse experiments. T.A. synthesized and provided the RH probe. A.G.Z., H.I., S.M.M., J.A., A.A., S.Y., E.V., T.A.C., E.N.Y., M.Ş.A., M.I.A., T.A., S.V., and E.E. contributed intellectually to data analysis, discussion, and manuscript preparation.

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Notes

The authors declare the following competing financial interest(s): A.G.Z., M.M., and E.E. have filed a patent application (patent application number 2023/000206) describing parts of this manuscript's research, which does not alter the authors' adherence to the policies on sharing data and materials presented in this study. The remaining authors declare no competing financial interests.

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Figure 1 (Agreement No. KC2935QK3H) and TOC (Agreement No. RD28 V0U23H) were created in BioRender. Eroglu, E. (2025) https://BioRender.com/4ay41gq.

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