

Monitoring of Endogenous Hydrogen Sulfide in Living Cells Using Surface-Enhanced Raman Scattering

Da-Wei Li,* Lu-Lu Qu, Kai Hu, Yi-Tao Long,* and He Tian

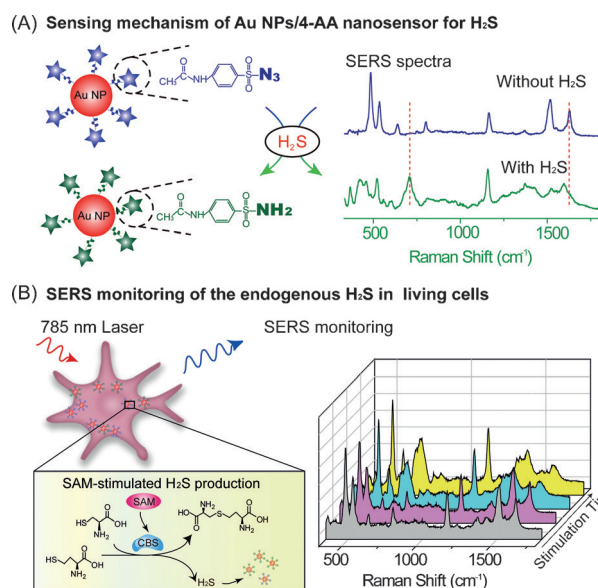
Abstract: Hydrogen sulfide (H₂S) has emerged as an important gasotransmitter in diverse physiological processes, although many aspects of its roles remain unclear, partly owing to a lack of robust analytical methods. Herein we report a novel surface-enhanced Raman scattering (SERS) nanosensor, 4-acetamidobenzenesulfonyl azide-functionalized gold nanoparticles (AuNPs/4-AA), for detecting the endogenous H₂S in living cells. The detection is accomplished with SERS spectrum changes of AuNPs/4-AA resulting from the reaction of H₂S with 4-AA on AuNPs. The SERS nanosensor exhibits high selectivity toward H₂S. Furthermore, AuNPs/4-AA responds to H₂S within 1 min with a 0.1 μM level of sensitivity. In particular, our SERS method can be utilized to monitor the endogenous H₂S generated in living glioma cells, demonstrating its great promise in studies of pathophysiological pathways involving H₂S.

Hydrogen sulfide (H₂S) has been recognized as a significant endogenous gaseous signaling molecule in living organisms,^[1,2] and it has been increasingly found to be closely associated with a number of pathophysiological processes.^[3–5] To comprehensively understand the physiological and pathological functions of H₂S, several methods have previously been explored for H₂S detection, such as colorimetry,^[6] electrochemical analysis,^[7] and gas chromatography.^[8] Unfortunately, these methods often require the destruction of specimens.^[9] By comparison, fluorescence detection is an attractive approach because of its suitability for in situ and noninvasive analysis.^[9–11] However, live-cell fluorescence detection usually suffers from the photobleaching and phototoxicity.^[12]

Surface-enhanced Raman scattering (SERS) has become a powerful analytical technique because it can provide molecular vibrational information;^[13] furthermore, SERS exhibits significant advantages over fluorescence methods, including resistance to photobleaching and phototoxicity, and narrow emission peaks for spectral multiplexing.^[14,15] SERS can also greatly enhance the detection sensitivity.^[16] As a result of these outstanding features, SERS has been

extensively applied in various fields, such as chemical analysis and biological sensing.^[16–18] Among its applications, SERS is typically used in probing organic molecules that readily attach to SERS substrates and have high Raman scattering cross-sections.^[15,16,19,20] However, the direct sensing of inorganic species with SERS remains a challenge because of their small Raman scattering cross-sections.^[21] To the best of our knowledge, SERS nanosensors for the selective and sensitive detection of intracellular H₂S have not been previously reported.

A potentially valid strategy to overcome this challenge that completely utilizes the advantages of SERS for H₂S detection is integrating SERS-active metal nanoparticles with molecules that have both high SERS responsiveness and identification ability toward the analyte.^[21] Inspired by the reduction of azides with H₂S under mild conditions,^[22,23] we herein report a novel SERS nanosensor fabricated by functionalizing gold nanoparticles with 4-acetamidobenzenesulfonyl azide (AuNPs/4-AA) to detect the endogenous H₂S in living cells (Scheme 1). We reasoned that, in the presence of H₂S, the azide groups of 4-AA molecules on AuNPs are transformed into amino groups, which causes SERS spectrum changes of AuNPs/4-AA. Thus, H₂S can be detected by observing changes in the SERS spectra. Moreover, in this way, the AuNPs/4-AA nanosensors were expected to enable the monitoring of endogenous H₂S, for example, produced by the



Scheme 1. SERS nanosensors for monitoring endogenous H₂S in living cells.

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cystathionine β -synthase (CBS) pathway in glioma cells under the stimulation of S-adenosyl methionine (SAM).

This new nanosensor of AuNPs/4-AA is approximately spherical with a diameter about of 60 nm (Supporting Information, Figure S1), which is efficient for Raman enhancement at near-infrared excitation (785 nm) in an aggregated state and suitable for the detection of biological samples.^[24,25] With the AuNPs/4-AA nanosensor in hand, we tested its SERS property and H₂S responsiveness in phosphate-buffered saline (PBS; 20 mM, pH 7.4). A clear SERS spectrum can be observed for the AuNPs/4-AA washed with ultrapure water, which indicates the successful functionalization of 4-AA molecules on AuNPs since SERS is a surface-selective technique. When NaHS (a common H₂S donor) is introduced to the AuNPs/4-AA, the SERS spectrum changes significantly (Figure S2). Specifically, the peak at 709 cm⁻¹ assigned to NH₂ wagging with mixing of ring in-plane bending appears, accompanied by the disappearance of the peak at 1626 cm⁻¹, which can probably be attributed to ring stretching with mixing of CO stretching and N₃ out-of-plane bending (Figure S2, Table S1). These changes reveal that the transformation of azides on AuNPs into anilines results from the reaction of H₂S with 4-AA, which may cause the response of NH₂ wagging to increase and make the N₃-related signal to decrease. Moreover, HPLC-MS analysis of the 4-AA solution in the presence of NaHS also confirms that the AuNPs/4-AA nanosensors are specifically responsive to the reductive reaction (Figure S3).

A temporal SERS observation of the AuNPs/4-AA nanosensors in the presence of NaHS indicates that the sensing reaction between the nanosensors and H₂S can be completed within 1 min (Figure S4), indicating that the SERS nanosensors are able to detect H₂S more rapidly than most of the reported probes for cellular H₂S (20 min–2 h).^[26,27] This may be because the electrophilic sulfonyl group renders the linked azido group more susceptible to the nucleophilic species of H₂S. Another possible reason is that AuNPs may play a catalytic role on the sensing reaction. As a result, the proposed nanosensors may be ideal for the real-time monitoring of H₂S in biological samples. Both the SERS spectra of the simple nanosensors and that of the nanosensors reacting with NaHS do not change significantly following over 20 h of storage, thus showing their good stability for sensing applications (Figures S5, S6).

The AuNPs/4-AA nanosensors are found to have high selectivity toward H₂S. When various analytes are mixed with the nanosensors, only NaHS induces a significant SERS peak at 709 cm⁻¹, whereas potential interferents, such as lipoic acid, GSH, L-cys, SCN⁻, SO₃²⁻, H₂O₂, O₂⁻, ClO₄⁻, HCO₃⁻, NO₂⁻, Br⁻, ClO⁻, Cl⁻, NO₃⁻, SO₃²⁻, SO₄²⁻, carotene, ascorbic acid, and uric acid, do not trigger obvious SERS signals at the same wavenumber (Figure 1A, pink histogram in Figure 1C). However, when NaHS is introduced into the test systems containing these interfering chemicals, the strong SERS peak at 709 cm⁻¹ appears (Figure 1B, yellow histogram in Figure 1C). The high selectivity of the AuNPs/4-AA nanosensors may result from both the specificity of the H₂S-sensing reaction and the fingerprinting feature of the SERS technique. On the other hand, AuNPs/4-AA reacting with H₂S

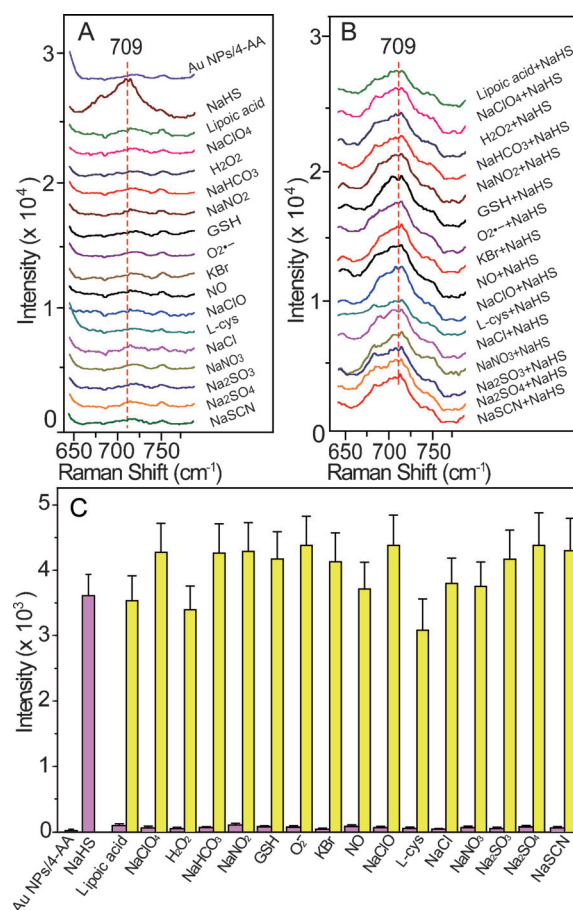


Figure 1. A) SERS spectra of AuNPs/4-AA (2.5 nm) in PBS (20 mM, pH 7.4) in the presence of various biologically relevant analytes. B) SERS spectra of AuNPs/4-AA in the presence of NaHS and other various biologically relevant analytes. C) SERS intensity at 709 cm⁻¹ based on the spectra in (A) and (B). Pink histogram: AuNPs/4-AA or AuNPs/4-AA with the indicated analytes. Yellow histogram: AuNPs/4-AA in the presence of both NaHS and indicated analytes. Data shown are 3.6 μ M for NaHS, 100 μ M for L-cys, and 500 μ M for other biologically relevant analytes. Each data point represents the average value from three SERS spectra. Error bars equal to the standard deviations.

rapidly and readily may be another important contribution to the specificity over other possible interferents including thiols (Figure S7), which even prevents GSH and L-cys at 2 mM from interfering with H₂S detection (Figures S8, S9). Moreover, the rapid sensing reaction may also be the reason why H₂O₂ and O₂⁻ do not obviously affect the detection although a relatively slow reaction is considered to occur between them and H₂S^[28] (Figures S10, S11). Therefore, the developed SERS nanosensors demonstrate a good potential for H₂S detection with minimal interference.

The AuNPs/4-AA nanosensors also demonstrate a dose-dependent response to H₂S. With increasing NaHS concentration, the ratiometric peak intensities of I_{709}/I_{1161} increases while I_{1626}/I_{1161} clearly decreases. Furthermore, there is an approximately linear relationship between I_{709}/I_{1161} (and I_{1626}/I_{1161}) and the logarithmic concentration of NaHS over the range of 0.12–84 μ M (Figure 2), and the detection limit (3 σ /

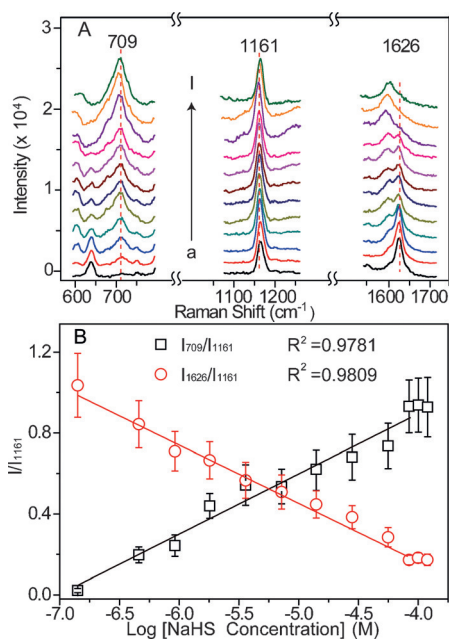


Figure 2. A) SERS spectra of AuNPs/4-AA (2.5 nm) in PBS (20 mM, pH 7.4) in the presence of NaHS with various concentrations (a to l: 0.12, 0.46, 0.92, 1.8, 3.6, 7.2, 14, 28, 56, 84, 100, and 120 μM). B) Plots of ratiometric peak intensities versus logarithmic NaHS concentration based on I_{709}/I_{1161} and I_{1626}/I_{1161} . Each data point represents the average value from three replicate SERS spectra. Error bars equal to the standard deviations.

slope) of AuNPs/4-AA sensors for NaHS is calculated to be 0.14 μM (upon I_{709}/I_{1161}) and 0.17 μM (upon I_{1626}/I_{1161}). I_{709}/I_{1161} and I_{1626}/I_{1161} are used because the peaks at 709 cm^{-1} and 1626 cm^{-1} are closely related with the sensing reaction while the peak at 1161 cm^{-1} is nearly independent on the reaction. Furthermore, because the usage of ratiometric peak intensities can help to prevent the influence of environmental factors,^[29] this SERS method has a good reliability at pH 4.0–9.0 (Figure S12) and its accuracy is also found to be comparable with the well-established spectrophotometric method (Figure S13).

The levels of H₂S endogenously produced in the brain from cysteine catalyzed by CBS decrease severely once CBS loses its activity, thus resulting in neurodegenerative diseases.^[30,31] However, SAM can regulate the activity of CBS to stimulate the generation of H₂S.^[32,33] We applied the AuNPs/4-AA nanosensors to monitor H₂S in human U251 MG glioma cells and rat C6 glioma cells under SAM stimulation. Bright-field microscopy images show that the two cell types both retained good morphology after the incubation of AuNPs/4-AA (Figure 3A1,A2), while dark-field microscopy (DFM) images indicate that the AuNPs/4-AA were endocytosed into the cells and mainly distributed in the cytoplasm region (Figure 3B1,B2). With the guidance of the DFM images, the positions of the AuNPs/4-AA can be localized, and SERS spectra from a single tested cell can be recorded readily. In the SERS spectra recorded from the cells stimulated by directly adding SAM (1.0 $\mu\text{g mL}^{-1}$) into the cell culture dishes, the characteristic peak at 709 cm^{-1} clearly appears, and I_{709}/I_{1161} becomes stronger with increasing

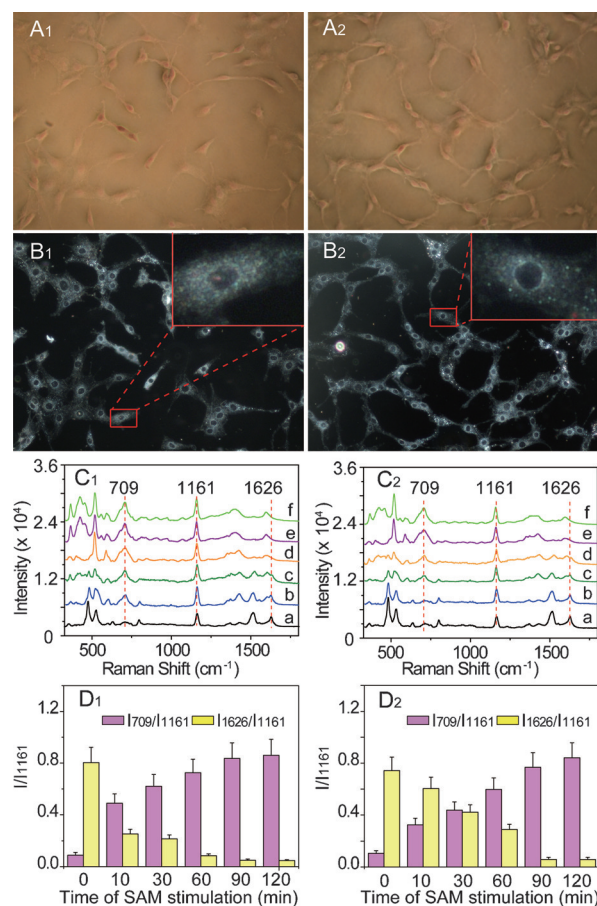


Figure 3. SERS monitoring of endogenous H₂S in living cells under SAM stimulation. (A1, A2) Bright-field images of rat C6 glioma cells and human U251MG glioma cells after 4 h incubation with AuNPs/4-AA. (B1, B2) DFM images of the corresponding rat C6 glioma cells and human U251 MG glioma cells containing AuNPs/4-AA. (C1, C2) SERS monitoring of rat and human cell-mediated H₂S with AuNPs/4-AA nanosensors under SAM stimulation for different times (a–f): 0, 10, 30, 60, 90, and 120 min. (D1, D2) Ratiometric peak intensities of I_{709}/I_{1161} and I_{1626}/I_{1161} versus time of SAM stimulation. The histograms are generated based on the spectra shown in (C1,C2). Each data point represents the average value from three replicate SERS spectra. Error bars equal to the standard deviations.

stimulation time, while I_{1626}/I_{1161} correspondingly decreases (Figure 3C1, D1, and C2, D2). The H₂S concentration was thus found to be improved by approximately 10-fold after 2 h stimulation, which confirms that SAM can activate CBS to enhance its catalytic capability to produce H₂S.^[31,32] In contrast, we can barely observe distinct signals and their variations at 709 and 1626 cm^{-1} for the negative control examples without stimulation (Figure S14), likely as a result of the CBS in glioma cells losing catalytic activity and therefore becoming unable to produce sufficient endogenous H₂S. Furthermore, the cell viability after the addition of AuNPs/4-AA was greater than 88% at concentrations from 1 to 10 nM (Figure S15), indicating the high biocompatibility of AuNPs/4-AA. Consequently, the developed AuNPs/4-AA nanosensors exhibit a powerful ability to study pathophysiological pathways that involve H₂S.

In summary, we have presented a novel SERS nanosensor for the rapid, selective, and sensitive detection of the endogenous H₂S in living cells. By functionalizing 4-AA on AuNPs, both H₂S responsiveness and SERS activity are integrated into the AuNPs/4-AA nanosensors. The specific transformation of the azide group of 4-AA into an amino group in the presence of H₂S results in SERS spectrum changes, thereby making the nanosensors suitable for the highly selective detection of H₂S. Using these SERS nanosensors, H₂S in solution can be rapidly detected within 1 min with a sub-micromolar sensitivity. In particular, we have also demonstrated that our SERS nanosensors can perform well for the real-time monitoring of H₂S generated in glioma cells under SAM stimulation, verifying the activating effect of SAM on the pathway of CBS-dependent H₂S generation. Furthermore, many reports have indicated that SERS nanoparticles have little toxicity to cells or tissues and that SERS methods may thus be very promising for in vivo investigations.^[34–36] Taken together, the proposed SERS strategy shows great potential for studies of pathophysiological processes in which selective and sensitive in vivo monitoring of intracellular H₂S is required.

Acknowledgements

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