Hydrogen sulfide (H\textsubscript{2}S) plays an important role in mammals as a signaling molecule. Recently, abnormal H\textsubscript{2}S concentration has been associated with several pathophysiological states, such as diabetes mellitus, hypertension, Alzheimer’s disease, and Parkinson’s disease. As regulating H\textsubscript{2}S concentration can be a very prominent way of developing new drugs, many researchers have paid great attention to H\textsubscript{2}S research. To understand the role of H\textsubscript{2}S in pathophysiology and develop H\textsubscript{2}S-based therapies, it is necessary to measure the exact concentration of H\textsubscript{2}S within biological systems. But, H\textsubscript{2}S is volatile and can be easily oxidized. Besides, the active sites for several biological effects of H\textsubscript{2}S are inside the cell. Therefore, there is a need for the development of new methods for the accurate and reliable detection of H\textsubscript{2}S within live cells. This review provides a summary of recent developments in H\textsubscript{2}S detection methods for live cell analysis.

1. Introduction

Hydrogen sulfide (H\textsubscript{2}S) is a biologically relevant gaseous signaling molecule, i.e., a gasotransmitter, collectively with nitric oxide (NO) and carbon monoxide (CO) [1–3]. The endogenous production and signaling capability of H\textsubscript{2}S in mammalian tissues were firstly demonstrated by Abe and Kimura in 1996, showing that H\textsubscript{2}S is an endogenous modulator in the central nervous system [4]. Since then, there has been a dramatic shift from the belief that H\textsubscript{2}S works entirely as an environmental toxin to the understanding that H\textsubscript{2}S plays an important role in organ function and homeostasis [5]. H\textsubscript{2}S has been revealed to take part in the regulation of various pathophysiological conditions within mammalian systems, such as vascular tone and blood pressure [6, 7], neurotransmission [8], angiogenesis [9], cardiac function [10], various leukocytic functions [11], penile erectile function [12], and gastrointestinal tract function [13]. In general, when the concentration of H\textsubscript{2}S in tissues or cells is high, H\textsubscript{2}S is regarded as a toxic substance and its oxidation products—persulfide, sulfite, thiosulfate, and sulfate—may give rise to cytotoxic effects through inhibiting mitochondrial cytochrome C oxidase and disrupting cell energy production, resulting in tissue inflammation or DNA damage [14]. On the other hand, H\textsubscript{2}S at low concentrations can lead to different effects on biological processes including DNA repair and metabolism, cellular division, regulation of cell cycle, modulation of protein kinase, and organization of cytoskeletal framework [15]. But the exact physiological role of H\textsubscript{2}S depends on the specific circumstance, its concentration, and the interplays with other signaling molecules—NO and CO.

Recently, there have been a few trials to classify the roles of H\textsubscript{2}S as “H\textsubscript{2}S-poor” and “H\textsubscript{2}S-rich” under pathophysiological conditions. First, there exist a few disease states where local or systemic H\textsubscript{2}S deficiency either due to inhibition of H\textsubscript{2}S biosynthesis and/or due to increased H\textsubscript{2}S consumption such as asthma, diabetic vascular complications, and aging [16]. Especially, H\textsubscript{2}S has been known to be associated to the pathogenesis of cardiovascular diseases including hypertension [17], atherosclerosis [18], and myocardial injury [19], and the severity of these diseases is negatively related to plasma H\textsubscript{2}S levels [20, 21]. Besides, the mean serum
H$_2$S level in preeclampsia patients was significantly lower than controls [22]. And Renieris et al. suggested that H$_2$S could be a potential marker for severity and final outcome of pneumonia by the SARS-CoV-2 coronavirus [23]. They showed that mortality was significantly greater among patients with a decrease of serum H$_2$S levels (a cut-off point of 150.44 µM) from day 1 to day 7 greater than or equal to 36%. Second, in some diseases like various forms of critical illness and multiple forms of cancer, H$_2$S biosynthesis is increased due to upregulation of H$_2$S-synthesizing enzymes [16]. But, there is a lack of understanding of the ideal level of H$_2$S in physiology and in therapy, as well as the normal concentration range of H$_2$S in circulation. One of the main obstacles is the insufficiency of an accurate and efficient detection method of H$_2$S for the screening and/or identification of possible H$_2$S donors and inhibitors.

H$_2$S is a colorless and flammable gas with the unique odor of rotten eggs [24]. As H$_2$S is a highly lipophilic molecule, it can easily penetrate the plasma membrane of all cells without any specific transporter or receptor [25]. Acute exposure to high amounts (more than 500 ppm) of H$_2$S can give rise to human death [14]. H$_2$S is a weak acid and easily dissolved in water with a solubility of about 80 mmol/L at 37°C [26]. Generally, it can dissociate into H$^+$ and hydrosulfide anion (HS$^-$), which may subsequently dissociate to H$^+$ and sulfide anion (S$^{2-}$) in aqueous solution. Because the two acid dissociation constants, pK$_{a1}$ and pK$_{a2}$, of this reaction are 6.9 and >11, respectively, H$_2$S is present in the approximate ratio of 20% H$_2$S and 80% HS$^-$ at physiological pH [27, 28]. Nevertheless, it remains unclear whether H$_2$S, HS$^-$, or both are biologically active. In addition to these free H$_2$S such as H$_2$S gas, HS$^-$, and S$^{2-}$, H$_2$S can exist in other bound sulfide pools in the biological systems including the acid-labile, alkaline-labile, and reducible sulfur, which are different from the conditions under which free H$_2$S is released [29, 30]. For example, acid-labile sulfide which is derived from iron-sulfur centers in mitochondrial enzymes [27] releases H$_2$S under an acidic condition (pH < 5.0). And H$_2$S is released from bound sulfane sulfur under reducing conditions including excess reduced glutathione (GSH), L-cysteine (Cys), and dithiothreitol (DTT) [27]. These complicated chemical species make it difficult to accurately measure free H$_2$S in biological systems. Actually, a lot of reports do not distinguish between the three most important biologic pools of labile sulfur: free H$_2$S, acid-labile, and DTT-labile sulfide, which may have completely different biological functions [31]. In addition, the volatility of H$_2$S adds complications to experiments [32]. For example, there are some published reports showing that half of H$_2$S can be rapidly released from culture medium in tissue culture wells within 5 min and in an even shorter time in a bubbled tissue bath [33, 34]. This may have in part contributed to large variations on the reported level of H$_2$S in plasma, tissues, and certain experiments [3, 35, 36]. During the past decade, several analytical methods such as methylene blue assay [37], gas chromatography [35], and sulfide-selective electrode [7] have been developed to detect H$_2$S in biological tissues or fluids. Earlier studies using the methylene blue assay reported that the H$_2$S level was 26–300 µM in mammalian plasma. However, later studies have shown that the high level of H$_2$S may be attributed to the use of a strong acid in the methylene blue method [38, 39], because H$_2$S can be released from acid-labile sulfur under a condition of a strong acid. Other methods have also been utilized to measure plasma H$_2$S levels in the rat: sulfide-selective electrode showed approximately 50 µM, and gas chromatography–mass spectrometry showed approximately 80 µM [40]. As gas chromatography is evidently sensitive and specific, we think that it can be helpful for the detection of low physiological H$_2$S levels. As a result, the large discrepancy among various reports may be attributed to the following reasons: (1) complications to experiments due to the intrinsic properties of H$_2$S including the instability of sulfide, its high volatility, its great susceptibility to oxidation, and its adherence to various materials (for example, glass); (2) improper experimental conditions such as the wrong release of sulfide out of some rubbers used; (3) there were no distinction between free H$_2$S, acid-labile, and DTT-labile sulfide; (4) different H$_2$S levels according to age, tissue, and species; and (5) different measuring methods.

To understand biological roles of free H$_2$S in health and disease state and develop H$_2$S-based therapies, it is necessary to detect the exact concentration of free H$_2$S within biological systems. As the active sites for various biological effects of H$_2$S are inside the cell, the H$_2$S level measured either in plasma or in homogenized tissue is not reflective of its cellular site of action [40]. To get more insight in their physiological roles, it is necessary to measure the H$_2$S level released from cells. However, it is difficult to apply conventional methods, such as methylene blue assay, gas chromatography, and sulfide-selective electrode, to live cells owing to their destructive nature. Therefore, there had been a need for the development of novel methods for the accurate and reliable detection of endogenous free H$_2$S within live cells.

Recently, a few studies regarding simple, facile, and inexpensive detection methods for the reliable detection of free H$_2$S in live cells have been reported. In this review, we have focused on recent developments in H$_2$S-sensing methods for live cells. We summarize the key characteristics of the analytical tools, cell types, experimental conditions for H$_2$S production, and H$_2$S concentration. In addition, the advantages and limitations of these methods are presented to provide a guideline for researchers to measure the H$_2$S levels released from live cells.

2. Spectrophotometric Methods for Live Cell Analysis

Among all the reaction-based spectrophotometric methods, the methylene blue method is the traditional standard. This method was introduced by Fischer in 1883 [41] and has been utilized for H$_2$S determination in many studies. As H$_2$S is very volatile and can be easily oxidized, sample preparation using Zn$^{2+}$ is generally required for the stabilization of H$_2$S. In this method, the acidic condition is generally employed to liberate H$_2$S from zinc sulfide complex. Subsequently, H$_2$S reacts with N,N-dimethyl-p-phenylene diamine (N,N-dpd) in the presence of an oxidizing agent (usually FeCl$_3$), producing methylene blue that strongly...
the agar layer. Then, the fl
layer, and H2S trapped in the agar as zinc sul
of Na2S ranging from 10 nM to 5 mM. They measured free H2S gas generated from live liver cancer cell line HepG2 cells a polystyrene microplate cover. The basic principle of H2S lizing a Ag-embedded Na2S, NaHS, diallyl disulfide, diallyl trisulfide, sodium thiosulfate (Na2S2O3), morpholin-4-ium 4-methoxyphenyl-morpholino-phosphinodithioate (GYY4137), and Lawesson’s reagent. Besides, Youness et al. [47] utilized this assay to measure the H2S levels released from MDA-MB-231 and MCF7 after silencing of CBS and cystathionine γ-lyase (CSE).

Although this method was the first report on the measurement of free H2S release in live cells utilizing the simple colorimetric method, the concentrations of SAM, Cys, and hCys used in this study were not physiological. But, this assay may be more helpful to explore the potential for Cys analogs and produgs to promote cytoprotection through the H2S pathway.

Zeng et al. [48] developed a colorimetric method for detection of H2S using gold (Au)/AgI dimeric NPs as optical probes. When Au/AgI NPs were reacted with H2S, AgI was changed to Ag2S, causing a shift in the plasmonic band of the AuNPs. The color and absorption changes were observed by naked eyes or measured by UV-vis spectroscopy (Figures 3(a) and 3(b)). In addition, the Au/AgI NPs were immobilized in agarose gels as test strips. These agarose gels were placed on the inner surface of the culture plate cover and then used for HepG2 cell culture. As a result, the H2S concentration was calculated as 167 nmol·h⁻¹·10⁻⁶ cells after treatment with Cys (2 mM) and PLP (0.5 mM) for 24 h (Figure 3(c)).

3. Fluorescence Detection and Imaging for Live Cell Analysis

Recently, small-molecule fluorescent probes have been attracted attention as an effective tool for detection and imaging of H2S in biological specimens such as tissues or cells due to their nondestructive property. The H2S-responsive fluorescent probes are mainly divided into four different categories depending upon their reaction types such as azide-to-amine reduction, nitro-to-amine reduction, copper sulfide precipitation, and nucleophilic addition [49, 50]. Early work in this field utilized the selective H2S-mediated reduction of azides and sulfonlalides, respectively, to develop first-generation reagents for fluorescence H2S detection [51]. Since then, Lin et al. [52] reported a family of azide-based fluorescent H2S indicators which had enhanced sensitivity and cellular trappability. In particular, sulfidefluor-7 acetoxymethyl ester enabled direct and real-time visualization of endogenous H2S release in live human umbilical vein endothelial cells under stimulation with vascular endothelial growth factor. And Yang et al. [50] developed a red-emitting fluorescent probe for H2S using the reduction of the azido group. This probe represented a
striking fluorescence enhancement (10-fold) with a large Stokes shift (125 nm), and the detection limit was 5.7 nM. They detected exogenous and endogenous H$_2$S in live HeLa cells.

Furthermore, Cheng et al. [53] developed a probe for the fluorescence switch-on detection of H$_2$S, by employing dinitrophenyl ether functionality as both a fluorescence quencher and an H$_2$S-reaction trigger. It was easily synthesized via nucleophilic substitution of 3,4-dinitrofluorobenzene by the BODIPY fluorophore 1. Its ability to image H$_2$S in live cells was demonstrated using HeLa cells and NaHS as the H$_2$S source. For specific and sensitive imaging of H$_2$S in the cellular lysosome, Wu et al. [54] developed activable fluorescence nanoprobe-based quantum dots. This nanoprobe consisted of p-amino thiophenol-capped AgNPs and thioglycolic-acid-stabilized quantum dots, called QD/AgNP nanocomplexes. The detection limit of this nanoprobe was 15 nM. And they showed high ability to enter into cellular lysosome in live HeLa cells.

Compared with "turn-on" fluorescent probes, ratiometric fluorescent probes have been proposed to be more accurate for detecting H$_2$S, independently of variables in quantitative analysis including variations of excitation intensity, environmental factors, light scattering, and concentration of probe [55]. An et al. [56] reported the quinoline quaternary ammonium salt derivative-based ratiometric fluorescent probe (referred to as QL-N$_3$). The QL-N$_3$ probe exhibited two fluorescence emission peaks at 525 and 605 nm with different excitation wavelengths of 385 and 521 nm, and the ratio between fluorescence intensities of two peaks was positively related with the H$_2$S concentration. This probe could image the changes in exogenous and endogenous H$_2$S in live HeLa cells.

Nevertheless, there are still several challenging issues in the development of fluorescent probes for H$_2$S as follows: (i) selectivity over interfering biothiols including GSH, Cys, and hCys; (ii) high sensitivity enough to detect the endogenously produced H$_2$S; (iii) fast response within a few minutes; (iv) biocompatibility including low toxicity, cell permeability, and intracellular stability; and (v) signaling in the biological optical window. Recently, Singha et al. [57] reported a two-photon fluorescent probe for H$_2$S which belonged to a Michael acceptor system. They approached the selectivity issue by optimizing the electronic and steric
interactions between biothiols and the probe, in addition to gaining very high sensitivity, biocompatibility, and fast response time.

Fluorescence-based detection provides excellent sensitivity, high selectivity, and real-time H$_2$S monitoring not only within living cells but also within subcellular organelles. Therefore, the progress of H$_2$S-specific fluorescence probes is regarded one of the fastest-growing areas in the field of H$_2$S biology [58]. Although fluorescence-based detection has attracted immense attention for detecting H$_2$S inside living cells, it is essential to use expensive instruments and special H$_2$S probes for live cell fluorescence detection. So, it may be difficult to utilize this method in many labs.

4. Surface-Enhanced Raman Scattering for Live Cell Analysis

Surface-enhanced Raman spectroscopy (SERS) is a promising ultrasensitive spectral analysis technique because of its high selectivity, based on molecular fingerprinting and sensitivity, even at single-molecule detection levels [59–61]. With strong electromagnetic fields and surface chemistry interactions...
enhancements, SERS can increase the original Raman signal to $10^6$ orders or more [62]. Recently, the SERS sensors have been utilized for the analysis of a variety of substances including DNA, protein, metal ions, and pesticides [63–65].

Li et al. [66] reported a novel SERS nanosensor fabricated by functionalizing AuNPs with 4-acetamidobenzenesulfonyl azide (AuNPs/4-AA) for detecting the endogenous H$_2$S in live cells (Figure 4(a)). The detection was performed with SERS spectrum changes in AuNPs/4-AA coming from the reaction of H$_2$S with 4-AA on AuNPs (transformation of the azide groups of 4-AA into amino groups). AuNPs/4-AA responded to H$_2$S within 1 min with a 0.1 μM level of sensitivity. Using SERS nanosensor, the H$_2$S concentration in living glioma cells was found to have approximately 10-fold increase after 2 h stimulation of SAM, confirming that SAM can activate CBS to improve its catalytic ability to produce H$_2$S. Besides, the viability of glioma cells after the addition of AuNPs/4-AA was higher than 88% at the concentration ranging from 1 to 10 nM, showing the good biocompatibility of AuNPs/4-AA (Figures 4(b) and 4(c)).

And Zhang et al. [67] proposed a smart SERS nanoprobe, Au core-4-mercaptobenzonitrile-Ag shell NP (Au@4-MBN@Ag), for detection of endogenous H$_2$S in live HepG2 cells. As sulfide in the solution selectively reacted with Ag to transform Ag$_2$S at room temperature, the SERS intensity of 4-MBN gradually decreased with increasing concentration of H$_2$S. It showed a good linearity in the sulfide concentration ranging from 0.05 to 500 μM, and a detection limit was 0.14 nM.

Though SERS has important advantages over fluorescence-based methods, such as resistance to photo-bleaching and phototoxicity, and narrow emission peaks for spectral multiplexing, it is still necessitated to develop H$_2$S-specific SERS probes due to the difficulty in direct sensing of inorganic species. In addition, SERS require expensive instrument—Raman spectrophotometer.

5. Paper-Based Colorimetric Assay for Live Cell Analysis

Paper-based sensors have received great attention in the development of point-of-care (POC) diagnostics owing to the simple fabrication, cost-effectiveness, and user-friendly characteristics. The distinct properties of paper which enable passive liquid transport and compatibility with chemicals or biochemicals are the main reasons on which paper is utilized as a sensing platform [68]. In addition, the white paper is suitable for colorimetric detection because it gives strong contrast with a colored substrate [69]. So, it enables readers to check the results with the naked eye.

Rosolina et al. [70] reported a bismuth-based disposable sensor using a wet, porous, and paper-like substrate coated with alkaline bismuth hydroxide, Bi(OH)$_3$. The alkaline,
wet coating helped the trapping of acidic H$_2$S gas and its reaction with Bi(III) species, forming colored Bi$_2$S$_3$ (yellow/brown). This sensor responded to $\geq 30$ ppb H$_2$S in a total volume of 1.35 L of gas. However, its alkalinity (pH 11) required special care in handling. And this sensor should be kept inside an inert gas to prevent neutralization by acidic CO$_2$ in air. Carpenter et al. [71] reported a new probe using the copper(II) complex of 1-(2-pyridylazo)-2-naphthol (Cu-PAN) on the same paper-like substrate. The reaction between H$_2$S gas and the copper complex led to a striking change in color from purple to yellow/orange. This color change was visible to the naked eye at the concentration as low as 30 ppb H$_2$S in a 1.35 L of gas which was considered as a typical volume of human breath. They suggested that the colorimetric paper probe is easy to fabricate, cost-effective, disposable, and a green alternative to the commonly used lead acetate test papers. Recently, Ahn et al. [72] developed a rapid and simple colorimetric paper sensor using an etching-resistant effect on Ag nanoprisms. The detection principle was that Ag NPRs on the paper reacted with H$_2$S gas to form Ag$_2$S on their surfaces, which induced etching-resistant Ag NPRs against Cl$^-$ ions. As a result, the color of Ag NPR-coated paper varied from yellow to purplish brown, depending on the concentration of H$_2$S gas after KCl treatment. This H$_2$S-sensing paper showed good sensitivity with a linear range of 1.03 to 32.9 $\mu$M H$_2$S and a fast response time of 1 min. The authors suggested that it could be utilized as a simple and reliable tool for on-site detection of H$_2$S gas for quality check of dietary supplements and human breath analysis. However, the H$_2$S-sensing papers

Figure 5: (a) Schematics of the preparation of Ag nanoplate-based H$_2$S-sensing paper and its principle of colorimetric and SERS dual-mode detection of H$_2$S. (b) The concentration of endogenous H$_2$S release from live LNCaP cells, varying with incubation times. (c) Evaluation of cellular toxicity of 5 mM L-cysteine (Cys) and 1 mM homocysteine (hCys) cotreatment in LNCaP cells. After incubation for 24 and 48 h, cellular toxicity was measured using water-soluble tetrazolium salt (WST) assay and expressed as a percentage of the control without Cys or hCys. Reprinted from [79] with permission from publisher.
Table 1: Some characteristics and H$_2$S levels of recently reported methods for the detection of free H$_2$S in live cells.

| Analytical methods for H$_2$S | Cell types | Experimental conditions for H$_2$S production | H$_2$S concentration | Advantages & limitations | Ref. |
|-------------------------------|------------|---------------------------------------------|----------------------|-------------------------|------|
| **Spectrophotometric method** |            |                                             |                      |                         |      |
| In situ methylene blue assay  | Rat A10 cells | 24 h incubation with 1, 3, and 5 mM of Cys and 1, 2, and 5 mM NAC | 40.67 ± 4.8 μM (with 3 mM Cys) | (Adv.) cost-effective & adaptable to most lab. settings (Limit.) low sensitivity, several complex steps & interferences with colored substances | [43] |
| Ag$_2$S@AgNPs in a layer-by-layer film | HepG2 cells | 2 h incubation with Cys and PLP (+Ag amplification for 2 h) | — | (Adv.) high sensitivity (10 nM) (Limit.) additional amplification time (2 h), except for the reaction time (2 h) | [44] |
| Ag-embedded Naion/PVP membrane | Rat C6 glioma cells | SAM treatment and with a combination of Cys (10 mM) and hCys (0.5 mM) for 48 h | 10.82 ± 1.66 μM | (Adv.) simple, facile, cost-effective, & adaptable to most lab. settings (Limit.) low sensitivity & treatment with high levels of substrates | [45] |
| Au/AgI dimeric NPs | HepG2 cells | 24 h incubation with 2 mM of Cys and 0.5 mM PLP | 167 nmol h$^{-1}$·10$^{-6}$ cells | (Adv.) good sensitivity (500 nM) (Limit.) long response time | [48] |
| Reduction of the azido group (λ$_{em}$ = 610 nm) | HeLa cells | Prestimulation with 100 μM of SNP (NO donor) for 60 min | — | (Adv.) high sensitivity (LOD 5.7 nM) (Limit.) only fluorescence cell imaging & long response time | [50] |
| Reduction of azide to amine (λ$_{em}$ = 526 nm) | HUVECs | Stimulation with VEGF (40 ng/mL) for 30 min | Intracellular fluorescence ratio $F_r/F_i$ ≈ 1.27 (vs. 1.07 of control) | (Adv.) good sensitivity (LOD 500 nM) (Limit.) long response time | [52] |
| Nucleophilic cleavage of the ether bond (λ$_{em}$ = 570 nm) | HeLa cells | 1 h incubation with NaHS (100, 200, and 300 μM) | — | (Adv.) good sensitivity (LOD 500 nM) (Limit.) only fluorescence imaging & long response time | [53] |
| H$_2$S-triggered disassembly of QDs/AgNP complexes (λ$_{em}$ = 530 nm) | HeLa cells | Pretreatment with 300 μM NaHS for 30 min | — | (Adv.) high sensitivity (LOD 15 nM) (Limit.) only fluorescence imaging & long response time | [54] |
| Reduction of azide to amino group | HeLa cells | 60 min incubation with Na$_2$S (100 μM) | The fluorescence intensity (red, 605 nm)/blue, 525 nm) ratio 2.416 (vs. 1.498 of untreated cells) | (Adv.) enhanced detection accuracy (ratiometric analysis) (Limit.) long response time | [56] |
| Surface-enhanced Raman scattering | Rat C6 glioma cells & human U251 MG glioma cells | SAM stimulation | Ratiometric Raman peak intensity $I_{1161}/I_{1161}$: about 10-fold increase after 2 h stimulation of SAM | (Adv.) good sensitivity (LOD 0.1 μM) & fast response time (1 min) (Limit.) needs specific SERS probe and expensive instrument | [66] |
mentioned above were not confirmed whether they worked properly at cell culture environment or in live cells.

Lee et al. [73] reported a new paper-based colorimetric assay by fabricating a 96-well microplate format for cell culture for sensing H₂S gas in live cancer cells. Microplate-like hydrophobic walls designed using AutoCAD were printed using a Xerox ColorQube 8570 N printer. Wax-printed paper (Whatman grade 1 chromatography paper) consisted of 96 circular reservoirs with a 4 mm detection zone. A PVP membrane containing Ag/NaF was coated on the H₂S detection zones. Finally, this paper-based colorimetric assay successfully measured the difference in endogenous H₂S level between live prostate cancer LNCaP and PC-3 cells, which showed differential expression of H₂S-producing enzymes. Though this paper-based assay was simple, inexpensive, and feasible, it could not measure the low concentration of H₂S released from LNCaP cells within 24 h.

6. Dual-Mode Detection for Live Cell Analysis

Although colorimetry-based sensing method is a simple and rapid technique for POC diagnostics and high-speed bioanalysis, the detection limit or sensitivity may be disappointing [74]. The low sensitivity can somewhat restrict the application of colorimetric methods for quantitative analysis of endogenous H₂S in live cells under shorter time. A dual-mode detection based on colorimetry and other sensing methods such as SERS, fluorescence, or wettability can improve the sensitivity of conventional colorimetric sensors, because they give two different types of output signals covering a wider detection range [75]. Gahlaut et al. [76] introduced a dual-mode H₂S detection combined colorimetric principle and wettability of Ag nanorod arrays on glass substrates. The surface color and water wetting properties of nanorods were found to be highly sensitive toward the H₂S gas environment (5 ppm of gas with an exposure time of only 30 s). Together with high sensitivity and selectivity, the response time was found to be significantly low (within 5 s). The authors suggested that this method could be applied for the future study of H₂S release from biosystems (live cells), as well as art conservation.

Zhong et al. [77] reported a colorimetric and near-infrared fluorescent probe (L) with a donor-π-acceptor structure derived from 4-diethylamino-salicylaldehyde and

| Analytical methods for H₂S | Cell types | Experimental conditions for H₂S production | H₂S concentration | Advantages & limitations | Ref. |
|----------------------------|------------|-------------------------------------------|-------------------|--------------------------|------|
| Paper-based colorimetric assay | LNCaP cells | 72 h incubation with 5 mM of Cys and 1 mM hCys | 17.48 ± 3.80 μM (72 h) | (Adv.) simple, low-cost, practical, & moderate sensitivity (LOD 1.4 μM) (Limit.) treatment with high levels of substrates | [73] |
| Dual-mode detection | MCF-7 cells | 30 min incubation with NaHS (10, 50, and 100 μM) | — | (Adv.) moderate sensitivity (LOD 3.09 μM) & fluorescence “off-on” response (Limit.) only fluorescence imaging | [77] |
| Colorimetry & SERS | LNCaP cells | Cys (5 mM) and hCys (1 mM) treatment for 8, 16, and 24 h | 0.144 ± 0.007 μM (8 h) 0.211 ± 0.007 μM (16 h) 2.45 ± 0.26 μM (24 h) | (Adv.) high sensitivity (LOD 15 nM for SERS detection & LOD 520 nM for colorimetry) (Limit.) treatment with substrates | [79] |
| Colorimetry & luminescence | HeLa cells | 30 min incubation with H₂S (200 μM) | — | (Adv.) high sensitivity (LOD 53.9 nM) (Limit.) only luminescence imaging | [80] |

1 Fᵢ: initial mean fluorescence intensity; Fᵢ: final mean fluorescence intensity. Cys: L-cysteine; NAC: N-acetylcysteine; VEGF: vascular endothelial growth factor; SNP: sodium nitroprusside; HUVEC: human umbilical vein endothelial cells; SAM: s-adenosyl methionine; hCys: homocysteine. PVP: polyvinylpyrrolidone; QDs: quantum dots; AgNP: silver nanoparticle; PLP: pyridoxal phosphate; A10: vascular smooth muscle cell; NHOF: normal human oral fibroblast.
2-(3-cyano-4,5,5-trimethylfuran-2(5H)-ylidene)malononitrile. A distinct color change of L solution from colorless to bluish-purple took place after treatment with H2S. Using this probe, they detected H2S vapor, H2S in water and wine samples, and H2S imaging in live MCF-7 cells. Besides, Paul et al. [78] reported a colorimetric and fluorescence turn-off probe 10-(4-azido phenyl)-5,5-difluoro-5h-dipyrrolo[1,2-c:1’,2’-f][1–3] diazaborinin-4-ium-5-uide, 1, for selective detection of H2S. The detection limit of this probe was 0.17 μM for H2S. They successfully detected exogenous H2S in live normal human oral fibroblast (NHOF) cells.

In addition, Ahn et al. [79] suggested a colorimetric and SERS-sensing system using Ag nanoplates on the paper. This dual-mode system could be helpful for detection of low concentrations of H2S in live cells because SERS could greatly improve the detection limit (Figure 5(a)). As a result, this simple paper assay could measure H2S with wider ranging from nano- to micromolar levels. And it was able to measure endogenous H2S in live LNCaP cells even at 8 h of incubation after cotreatment with Cys (5 mM) and hCys (1 mM) (Figure 5(b)). Besides, the viability of LNCaP cells was greater than 90% for 48 h, indicating good cell proliferation of live cells (Figure 5(c)).

Liu et al. [80] reported a ruthenium (Ru) (II) complex-based probe for colorimetric and luminescent detection and imaging of H2S in live cells and organisms. This Ru(II) complex was yellow color and nonluminescent in aqueous solution. But, when it reacted with H2S, the color of the solution changed from yellow to pink for colorimetric analysis and the emission intensity was about 65-fold increased for

**Figure 6:** (a) Schematic diagram of the H2S detection process in live cells. (b) Comparison plot of sensitivity versus accessibility & usability of various analytical methods for detection of H2S in live cells.
luminescent analysis. As this probe had low cytotoxicity and good permeability to cell membrane, it could be utilized for luminescence imaging of H$_2$S in live HeLa cells.

Until now, we described the recent developments in H$_2$S detection methods for live cell analysis. Table 1 summarized some characteristics, H$_2$S levels, advantages, and limitations of recently reported methods for the detection of free H$_2$S from live cells. And Figures 6(a) and 6(b) show a schematic diagram of the H$_2$S detection process in live cells without causing any destruction to the cells and a comparison plot of sensitivity versus accessibility of various analytical methods, respectively.

7. Conclusions

In this review, we have summarized the methods for the detection of H$_2$S in live cells without causing any destruction to the cells. To measure H$_2$S level in conventional biology, an indirect method that analyzes the expression of H$_2$S synthase, such as CBS and CSE, in cell lysate or tissue homogenate using Western blot analysis is widely used. But the total analysis time for the Western blot from cell seeding is approximately 7 days including all the necessary processes such as treatment of substrates and cell lysis. To eliminate or reduce the complicated and labor-intensive analytical approach, several methods that are simple, efficient, and reliable for detection of H$_2$S in live cells have been developed. Microplate cover-based and paper-based colorimetric assays utilizing Ag/Nafion/PVP membrane can quantitatively analyze the endogenous H$_2$S levels in live cancer cells, without expensive instruments and special H$_2$S probes. Although these colorimetric assays are simple, easy to use, and cost-effective, they still have the limitation of low sensitivity. Recent developments in fluorescent probes for reactive sulfur species can further facilitate analysis based on fluorescence bioimaging technology. But, there are still challenging issues including high sensitivity and selectivity in the presence of many interfering biomolecules, water solubility, and low cytotoxicity. And SERS detection using H$_2$S-responsive SERS probe shows great promise for the real-time monitoring of H$_2$S produced in live cells, though it requires a Raman spectrophotometer. The dual-mode detection based on colorimetry and other detection methods such as SERS or fluorescence can improve the sensing performance such as high sensitivity and wide detection range, as well as easy to use. And further work for developing the highly sensitive, specific, biocompatible, and reproducible detection method is required to measure free H$_2$S in live cells in the absence of additional substrates or stimulator.

Conflicts of Interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

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