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Measurement of low concentration and nano-quantity hydrogen sulfide in aqueous solution: measurement mechanisms and limitations

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Abstract
To measure hydrogen sulfide (H_2S) level in biological samples *in vivo* on a real-time basis with high resolution is greatly needed for advancing our understanding of the biological role of H_2S. Traditional H_2S measurements usually need large tissue samples and complex procedures. However, H_2S concentration is very low in human bodies and the tissue sample is limited for medical treatment purposes. There is a need to develop a new paradigm for the real-time measurement of a trace amount of hydrogen sulfide with a small amount of tissue samples or *in vivo*. We previously reported a method to measure low concentration H_2S solution using carbon nanotubes and the fluorescence spectra of Raman and confocal laser scanning microscopes. We obtained that the measurement resolution with a confocal laser scanning microscope is higher than that with a Raman microscope; in particular, 10 μM concentration difference can be detected with a confocal laser scanning microscope. In this paper, we present the underlying mechanism and limitation of this method together with other traditional methods based on the theoretical analysis, which leads to the finding of further research on the measurement of low concentration and nano-quantity H_2S solution.

Keywords: hydrogen sulfide, carbon nanotubes, mechanism, measurement resolution

(Some figures in this article are in colour only in the electronic version)

1. Introduction

H_2S is a colourless and flammable gas at room temperature. Its molecular structure is the same as that of water. In other words, the sulfur atom has its own set of electrons, while the hydrogen has one electron for each of its two atoms. When one sulfur atom and two hydrogen atoms are properly combined, the sulfur atom and the hydrogen atoms share all the electrons available, creating a bond among the three different atoms

In mammals, H_2S can be detected in blood, brain, lung, heart, liver, spleen and kidney [4–7]. Its physiologically relevant circulation concentration is around 30–100 μM [4–7]. Traditionally, H_2S is considered to be toxic. Recent studies have, however, revealed that this molecule of gas in the

cardiovascular system may play a role as a vascular dilator [4]. Whether H_2S plays a physiological, pathophysiological or even a toxicological role depends on its *in vivo* level. As such, the measurement of H_2S in mammalians with nano-quantity resolution in an *in vivo* and real-time manner becomes an important subject.

The existing methods for the measurement of H_2S in the biological system include chromatography, spectrophotometry and sulfide ion-specific electrode. These methods were originally derived from the measurement of sulfide in polluted air and water samples, and when applied to biological systems they appear to have a common procedure: (1) tissues are taken out of animal bodies and homogenized; (2) the homogenized tissues are reacted with L-cysteine to generate hydrogen sulfide as the active CBS (cystathionine β-synthase) which is the predominant H_2S-generating enzyme in the brain and nervous system [8] or CSE (cystathionine γ-lyase) which is mainly expressed in vascular smooth muscles [5] in the homogenization. Clearly, these methods require large tissue samples and therefore are not promising for real-time *in vivo* measurement of H_2S in mammals.

We previously reported a method towards the real-time *in vivo* measurement of H_2S in blood using carbon nanotubes and the fluorescence spectra of the confocal laser scanning microscope and Raman microscope [9]. We also showed that the measurement resolution with the confocal laser scanning microscope was higher than that with the Raman microscope; particularly 10 μM concentration of H_2S in the H_2S solution can be detected with the confocal laser scanning microscope, but not by the Raman microscope. In this paper, we present the underlying mechanism for this method and an analysis of limitations of this method along with those of the three existing methods.

This paper is organized as follows. Section 2 presents the mechanisms of the method we developed. We also discuss the reason that the confocal laser scanning microscope is better than the Raman microscope in our approach. In section 3, we discuss the mechanisms behind the three traditional methods, namely chromatography, spectrophotometry and sulfide ion-specific electrode, respectively. We will show their limitations as well. Section 4 concludes this paper with a discussion of some future works.

2. The carbon nanotube based method: its mechanism and limitation

2.1. Carbon nanotubes

Our method was to use carbon nanotubes (CNTs) to interact with H_2S solutions and then to use either a Raman or a confocal laser scanning microscope to get the spectra of fluorescence from the CNTs before and after they interact with the H_2S solutions. Therefore, there are two mechanisms for this method: one related to the CNTs and the other related to the Raman or confocal laser scanning microscope.

Carbon nanotubes have been the focus of research since their discovery in the early 1990s [10]. A single-wall carbon nanotube is analogous to a sheet of graphite rolled into a cylinder closed at either end with caps containing pentagonal rings and a multi-wall carbon nanotube is several sheets of rolled graphite. Depending on the twist direction of the graphite sheet, nanotubes can be metallic or semi-conductive. Due to their special structure, perfect carbon nanotubes are light, flexible and thermally stable [11]. Carbon nanotubes can be produced by different methods and under different conditions. However, the uniform pore size distribution, high surface area and excellent electronic properties make carbon nanotubes promising materials for hydrogen sulfide adsorption. Some defects in carbon nanotubes, which modify the electronic density around defects, need more energy to restore the broken bonds. This leads to an increase in the adsorption with the carbon nanotubes.

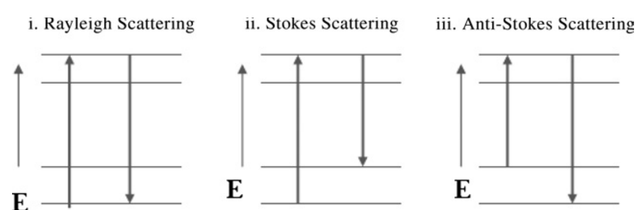

Figure 1. Simplified Raman energy level diagram [14].

The main idea of using carbon nanotubes comes from the fact that H_2S can be adsorbed by activated carbon. Adsorption of H_2S onto activated carbon surfaces included both reversible and irreversible components. Reversible adsorption is affected mainly by the pore structure, and pore filling is likely the dominant mechanism [1]. The irreversible adsorption of H_2S is affected by chemical reaction. Dry H_2S and oxygen do not react at ambient temperature, but react at temperatures above 200 °C. However, in the presence of activated carbon, H_2S can react with oxygen at low temperatures and produce sulfur and water [2, 3]. The irreversible amount of H_2S increases with increasing surface area [1].

When carbon nanotubes are in the H_2S solution, a thin water film is formed. Oxygen is dissolved in the film and oxygen molecules are adsorbed on the carbon nanotubes and become reactive points. The H_2S that reaches the carbon nanotube is also dissolved in the film and H_2S molecules are partly dissociated into protons and hydrosulfide ions. The latter reacts with the oxygen at its reactive point to form hydroxyl ions and sulfur which is adsorbed on the carbon nanotube. The protons neutralize the hydroxyl ions and produce water. The fluorescence spectrum of sulfur on a carbon nanotube can be measured by a Raman and/or a confocal laser scanning microscope when properly selected excitation and emission wavelengths are used.

2.2. Raman

The principle of Raman spectroscopy is shown in figure 1. It sends monochromatic light (only one colour, not a mixture) to the sample. The incidental photons are absorbed, and their energy is used for creating scattered photons and for creating (Stokes process) or destroying (anti-Stokes process) vibrations in the sample. The light from the sample is collected such that the wavelength of the laser is filtered out and those in a certain spectral window away from the laser line are dispersed onto a detector [12, 13].

Raman scattering is typically very weak, and as a result the main difficulty with Raman spectroscopy is to separate the

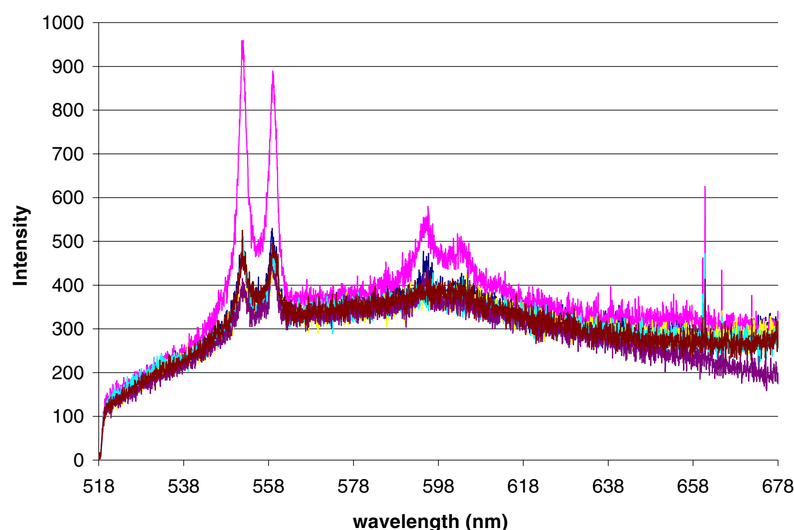

Figure 2. Fluorescences of different concentrations (0 μM, 10 μM, 20 μM, 30 μM, 40 μM and 50 μM) of H_2S in carbon nanotubes by Raman spectroscopy.

weak inelastically scattered light from the intense Rayleigh scattered light. Currently, a laser is used as the light source in the Raman microscope to increase the resolution. However, the intrinsically weak Raman signal needs high laser powers (typically > 10 mW) and is often overwhelmed by the fluorescence background of the sample. In a Raman microscope, if the excitation wavelength is larger than 300 nm, the fluorescence background can significantly affect the signal. The UV laser source (<300 nm) can enhance the intensity, and therefore increase the ability to discriminate the difference in low concentrations. For example, the enhancement of a 244 nm UV laser is 20 times that of a 514 nm laser.

Raman spectrometers typically use holographic diffraction gratings and multiple dispersion stages to achieve a high degree of laser rejection. The step of the dispersion stages of the Raman microscope we used is about 5 μm, which is too large for nano-size carbon nanotubes and may cause random errors. The sensitivity of the CCD camera used in Raman microscope is another important source which can affect high resolution signal collection.

Figure 2 demonstrates this influence. The samples used were purified carbon nanotubes prepared by microwave plasma enhanced chemical vapour deposition (MPECVD). These purified carbon nanotubes (1.6 mg) were immersed in 300 μl distilled water, 10 μM, 20 μM, 30 μM, 40 μM and 50 μM H_2S solutions, respectively, for 2 min at room temperature; 5 μl of each sample was dropped on glass slides and was dried at room temperature. The dried carbon nanotubes on the glass slides were used for measurement. The result is shown in figure 2. From the figure, it is hard to separate six curves. This means the Raman microscope (Renishaw system 2000) with the 514 nm excitation wavelength and 518–700 nm emission wavelengths (these wavelength were selected with the use of the confocal microscope, which will be discussed later) is not able to distinct 10 μM difference in H_2S concentration.

2.3. Confocal laser scanning microscope

Unlike other microscopes, a pinhole is used in a confocal laser scanning microscope (figure 3) such that a laser beam

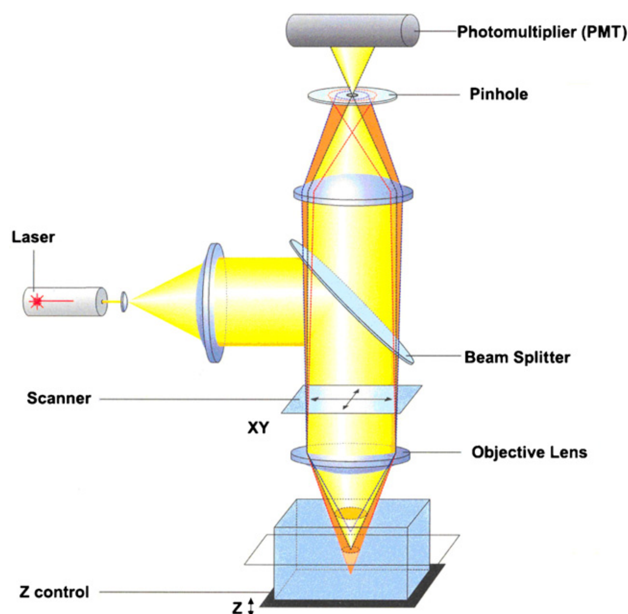

Figure 3. Principle of confocal laser scanning microscope (Zeiss LSM 510 Meta Manual).

is focused by an objective into a small focal volume within a specimen. A mixture of emitted fluorescent light and reflected laser light from the illuminated spot is then collected by the objective. A beam splitter separates the light mixture by allowing only the fluorescent light to pass through and reflecting the laser light. After passing a pinhole, the fluorescent light is detected by a photomultiplier (PMT) which transforms the light signal into an electrical one and is recorded by a computer.

In laser scanning microscope, a fluorescent specimen is illuminated by a point laser source. The scanning laser point source is not an infinitely small point but a three-dimensional diffraction pattern determined by the optical system, including the numerical aperture of the system's objective and the wavelength of the laser light used. This limits the resolution of

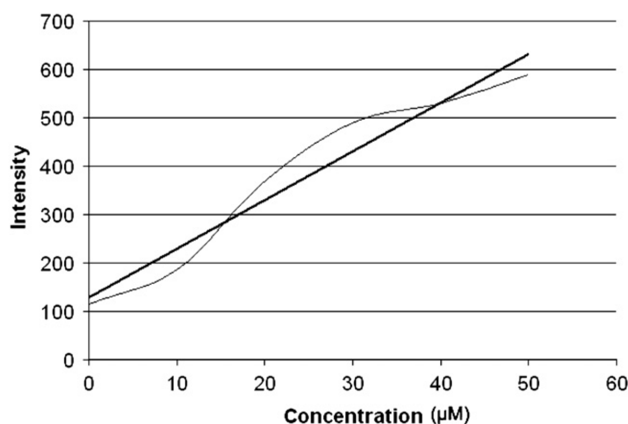

Figure 4. The intensity versus the concentration of H_2S with purified carbon nanotubes.

conventional optical microscopes. However, with the confocal laser scanning microscope it is possible to overcome this resolution limit as only a small volume element is detected at a time. The resolution limit in a confocal laser scanning microscope depends not only on the illumination but also on the creation of enough detectable photons. By using the light creation process with much lower probabilities of occurrences such as second harmonic generation, the volume of area is reduced to a small region of the highest laser illumination intensity which results in a significant improvement in the lateral resolution. By using a more sensitive photo-detector, the signal-to-noise ratio can be improved. However, the signal-to-noise ratio still limits further higher resolution. Increasing excitation intensity can possibly enhance the signal-to-noise ratio but high intensity of the laser beam may be too strong to bleach the sample. Such a dilemma is a challenge to further improving the measurement of H_2S using a confocal laser scanning microscope.

Using the same samples as described with the Raman microscope and confocal laser scanning microscope (ZEISS LSM 510 META), with different excitation wavelengths in lambda mode, the 10 μM difference in H_2S concentration can be detected by an optimized excitation wavelength at 514 nm and emission wavelength at META channel from 539 nm to 753 nm (figure 4). From this figure, the intensity versus the concentration of H_2S solution appears to be approximately linear and the emitted fluorescence intensity from the carbon nanotubes increases with increasing concentration of hydrogen sulfide solution.

3. The traditional methods: mechanisms and limitations

3.1. Chromatography

Chromatography, including gas chromatography, liquid chromatography, ion exchange chromatography and affinity chromatography, is a measurement technique used for analysing and/or separating mixtures of chemical substances. The basic working principles are described in the following [15]: a sample of the mixture to be analysed (the analyte) is applied to some stationary fixed material (the adsorbent) and then a second material (the eluent) is passed through or over the stationary phase. The compounds contained in the analyte are then partitioned between the stationary adsorbent and the moving eluent. As different materials adhere to the adsorbent with different forces, some adhere to the adsorbent more strongly and therefore move through the adsorbent more slowly when the eluent flows over them, while other components of the analyte are less strongly adsorbed on the stationary phase and move along more quickly with the moving eluent. Therefore, as the eluent flows through the column, the components of the analyte will move down the column at different speeds and therefore separate from one another.

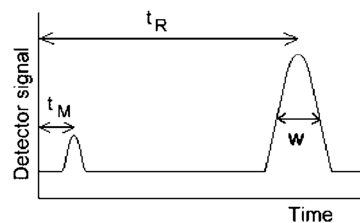

Figure 5. Distribution of analytes between phases.

The resolution of chromatography is defined as [16]

$$R = (N^{1/2}/4)((\alpha - 1)/\alpha)((1 + k)/k) \tag{1}$$

where $N = 5.55t_R^2/w^2$; t_R is retention time which is the time between the sample being injected and the time the analyte peak reaches a detector at the end of the column. Each analyte in a sample will have a different retention time; w is the half width of the peak (figure 5).

Retention factor $k = (t_R - t_M)/t_M$ and t_M (figure 5) is the time for the mobile phase to pass through the column of the chromatography. Further, selectivity factor $\alpha = k_B/k_A$, which describes the separation of two species (A and B) on the column. Note that here species A elutes faster than species B. The selectivity factor is always greater than 1.

The concentration difference that can be detected is defined as the interval between two adjacent peaks when the amplifier attenuation is set such that the smaller peak is at least 50% of full scale. The limit is achieved when $R = 1.5$. For example, the measurement of H_2S in air by ion chromatography has the working range of 20–500 μM for a 20 l air sample [17].

3.2. Spectrophotometry

Spectrophotometry is widely used to measure trace amounts of H_2S. It is also known as the methylene-blue method in analytical chemistry as the dye methylene blue will be formed when the H_2S solution reacts with ferric chloride ($FeCl_3$) and *N*,*N*-dimethyl-*p*-phenylenediamine. Absorbance of the dye in solution is then measured by the spectrophotometer. When monochromatic light (light of a specific wavelength) passes through a solution there is usually a quantitative relationship (Beer's law) between the solute concentration and the intensity of the transmitted light, that is,

$$I = I_o \times 10^{-kc\ell} \tag{2}$$

where I_o is the intensity of transmitted light using the pure

Table 1. Comparison of the current methods for H_2S measurement.

Measurement methods	Advantages	Disadvantages
Spectrophotometry	Widely used Less expensive	Long experimental time (1.5 days) *In vitro* measurement
Chromatography	Accurate detection Separate H_2S from mixtures of chemical substances	Expensive for the first setup *In vitro* measurement
Sulfide ion-specific electrode	Easy to operate Low cost for the initial setup	Needs to be calibrated before every measurement

solvent, I is the intensity of the transmitted light when the coloured compound is added, c is the concentration of the coloured compound, ℓ is the distance the light passes through the solution and k is a constant.

If ℓ is a constant, as is the case with a spectrophotometer, Beer's law can be written as

$$I/I_o = 10^{-hc} = T \quad (3)$$

where T is the transmittance of the solution and h is a constant. We can also write this equation as

$$-\log T = hc = K = \text{optical density} \quad (4)$$

$$\Delta c = \Delta K/h. \quad (5)$$

The minimum concentration of H_2S that can be measured is related to the optical density changes that can be detected by the instrument. Photoacoustic spectroscopy of H_2S converted to methylene blue has greater sensitivity than standard spectrophotometric methods (NIOSH 1979). By maximizing the instrument response to the 750 nm peak, it is possible to achieve a detection limit of 3×10^{-4} μM when samples are collected at 2.0 l min^{-1} for a 1 h period.

3.3. Sulfide ion-specific electrode

First, a typical sulfide calibration curve is obtained. This means that electrode potentials of standard solutions (the concentrations of the standard solutions were known before the measurement) are measured and plotted on the linear axis against their concentrations on the log axis. The linear axis (y-axis) denotes the milli-volt values and the log axis (x-axis) represents the concentration values of the solutions. Then, the electrode potential of the sample solution is recorded. From this linear calibration curve, an unknown sample concentration with its milli-volt values can be obtained. The calibration procedure for H_2S is very complicated because sulfide ion-specific electrodes are only sensitive for the fully dissociated form of sulfide. However, the equilibrium between H_2S, HS^- and S^{2-} is pH dependent such that the calibration needs to be carried out in a well-buffered system of known pH, and a complete lack of oxygen [18]. The accuracy of standard solutions, usually measured by the methylene-blue method [19], depends on the measurement method. Typically, the ion-specific electrode has a linear response range between 10^{-1} M and 10^{-5} M and detection limit on the order of 10^{-5}–10^{-6} M. However, the observed detection limit is often affected by the presence of other interfering ions or impurities [20]. Recently, Dennis *et al* reported measurement of very low sulfide concentration (0.5 μM); however, their method needs steady state concentrations with a modified sulfide ion-specific electrode [21].

The advantages and disadvantages of each method are summarized in table 1. All these methods generally require bulky samples and are invasive and off-line, and thus they are not qualified for *in vivo* and non-invasive measurement.

4. Conclusion

(1) Traditional methods usually need bulk samples and take a long time in H_2S measurement, so they are not promising for *in vivo* and real-time measurement.
(2) The CNT-based method can achieve nano-quantity and low H_2S concentration measurement with conventional energy beams such as those in the confocol and Raman microscopes.
(3) Although currently 10 μM H_2S concentration can be measured using the CNTs with a confocal microscope, the mechanism behind this method does not limit much lower concentrations. Using a high energy beam instrument such as deep x-ray, much lower concentrations could be achieved.
(4) CNT-based methods need very little tissue, so they have promise for clinical use and for further development for *in vivo* and real-time H_2S measurement.

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