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Measurement of low concentration and nano-quantity hydrogen sulfide in sera using unfunctionalized carbon nanotubes

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Abstract

Hydrogen sulfide (H₂S) is produced in small amounts by certain cells in the mammalian body and has a number of biological functions. H₂S gas naturally produced by the body is not simply a toxic gas; it could be a vascular dilator and play a physiological role in regulating cardiovascular functions. In order to know the effects of H₂S, it is necessary to accurately know its concentrations in the body. Conventional measurement methods have their limitations concerning the small amount and low concentration of H₂S in the body. A new paradigm of using carbon nanotubes in H₂S measurement expresses its potential. However, the influence of proteins in the mammalian body must be studied in the measurement of H₂S by carbon nanotubes. In this paper, we demonstrate a successful measurement of low concentration (20 μM) and nano-quantity (0.5 μg) H₂S in the serum by using carbon nanotubes and further with the fluorescence of confocal laser scanning microscopy and the luminescence of Raman microscopy. Statistical analysis of the experimental data shows that the relationship between concentrations and intensities is linear, which thus makes the carbon nanotube sensor highly promising for the measurement of H₂S in sera.

Keywords: hydrogen sulfide, carbon nanotubes, serum proteins

1. Introduction

Hydrogen sulfide (H₂S) is a colorless, toxic, flammable gas that has the foul odor of rotten eggs [1, 2]. The molecular weight of H₂S is 34.08 g. Density of H₂S is 1.5392 g L⁻¹ at 0 °C, 760 mm Hg and its water solubility is 3980 mg L⁻¹ at 20 °C. Hydrogen sulfide has a structure similar to that of water. However, sulfur is not nearly as electronegative as oxygen, so hydrogen sulfide is not nearly as polar as water. Because of

this, comparatively weak intermolecular forces exist between the H₂S molecules, and their melting and boiling points (vapor pressure: 15 600 mm Hg at 25 °C; boiling point: -60.33 °C) are much lower than those of water. Aqueous solutions of H₂S are not stable. Oxygen causes H₂S solutions to form elementary sulfur, and the solutions become turbid rapidly [3]. H₂S can poison several vital systems in the mammalian body; among them the nervous system is the most affected.

H₂S can be measured in the mammalian body. For instance, the physiological concentration of H₂S in brain tissue

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has been reported to be 50–160 μM [4, 5]. Recent studies have shown that some cells in the mammalian body such as vascular tissues can generate measurable amounts of H_2S [5, 6]. Stipanuk *et al* [7] used rats in the weight range from 190 to 269 g, and reported that H_2S can be produced at 0.01–1.67 $\mu\text{mol} (\text{min g})^{-1}$ in the skeletal muscle, 0.018–1.42 $\mu\text{mol} (\text{min g})^{-1}$ in the brain, 0.037–4.11 $\mu\text{mol} (\text{min g})^{-1}$ in the heart, 0.25–2.26 $\mu\text{mol} (\text{min g})^{-1}$ in the kidney and 0.645–3.78 $\mu\text{mol} (\text{min g})^{-1}$ in the liver. Produced from cysteine by various enzymes, H_2S is hypothesized to fulfill a physiological role in regulating cardiovascular functions which are distinctive from its toxicological effect. Apart from functioning as a vascular dilator [6, 8–10] in the cardiovascular system, H_2S in the mammalian body has a number of other biological functions. Whether H_2S plays a physiological, pathophysiological or toxicological role depends on its *in vivo* level. The different concentrations of H_2S may play different roles in the mammalian body [11–13]. Since the concentration of H_2S in the mammalian body is typically very low (about 40 μM), the measurement of low concentrations of H_2S in the mammalian body is highly desired.

Current methods for the measurement of H_2S include chromatography [14], spectrophotometry [15] and sulfide ion-specific electrode [16]. These methods were originally developed from the determination of sulfide in a polluted air or water sample and were applied to biological samples. Each of these methods appears to have a relatively complex procedure and certainly needs large amounts of tissue samples. Furthermore, these methods are intrusive and in an off-line measurement manner.

Recently, we have reported that low concentration (down to 10 μM) and nano quantity (down to $1.7 \times 10^{-2} \mu\text{g}$) of H_2S can be measured in water solutions using unfunctionalized carbon nanotubes with the fluorescence spectrum by the Zeiss LSM 510 Meta microscope [17]. This method predicts a further potential to measure low concentration and nano-quantity H_2S in mammalian blood or serum. This is, however, a challenging problem, as the proteins in the serum may ‘disturb’ the measurement of H_2S using carbon nanotubes; in other words, the specificity of measurement of H_2S can be challenged.

The reason that LSM 510 Meta was employed to acquire the fluorescence spectrum is that this instrument has a pinhole to control the focus point and to collect the reflected light by the lens more effectively; whereas a regular fluorescence microscope can only focus on a volume, and the reflected light is not collected by the lens effectively. Raman luminescence can also be used for the same purpose but is not as good as the LSM fluorescence [18].

In the study presented in this paper, we further extended our work reported in [17] to the measurement of hydrogen sulfide in the serum using unfunctionalized carbon nanotubes. Our hypothesis was that unfunctionalized carbon nanotubes can adsorb the hydrogen sulfide in the serum under the presence of proteins, and the influence of proteins on the fluorescence measurement seems to be statistically insignificant. In the following discussion, CNT refers to the unfunctionalized carbon nanotube if without further note.



Figure 1. A typical TEM image of carbon nanotubes.

2. Materials and methods

2.1. Preparation of CNTs and measurement of H_2S

Three experiments were performed to test our hypothesis. In all these experiments, multi-wall CNT samples were used and they were prepared by using a microwave plasma enhanced chemical vapor deposition (MPECVD) reactor in a gas mixture of hydrogen and methane and were purified at 450 $^{\circ}\text{C}$ until there was no further weight loss with the CNT in the process. A typical transmission electron microscope (TEM) image of these CNTs is shown in figure 1. TEM observation shows that the outer diameter of a nanotube is about 25 nm and the inner diameter is about 5 nm. Note: the CNTs used in the following experiment were all this type of unfunctionalized CNTs.

The concentration of H_2S in the serum was measured by a sulphide sensitive electrode (Model 9616, Orion Research, Beverly, MA, USA) on a Fisher Accumet AR50 pH meter (Fisher Scientific, Pittsburgh, PA) following the manufacturer's instruction. Standards are prepared from the Na_2S stock solution, which is freshly prepared on the day of the measurement [19].

2.2. Measurement of proteins in the serum

The serum sample was separated into two equal parts (each 300 μL): one was used as a control sample (i.e., it was not treated by the CNT) and the other was used as a test sample. The test sample was further made by adding 1.6 mg CNTs and left overnight. After that, the serum which served as a test sample was taken from the top of the treated serum. The control serum sample and the test sample were measured using spectrophotometry and electrophoresis, respectively, to see if there is any change in protein quantity between the control serum sample and the test sample.

Serum proteins which were measured are: total protein, albumin, alpha1-globulin, alpha2-globulin, beta globulin and gamma globulin. The total protein and albumin protein were measured colorimetrically with a spectrophotometer,

specifically the synchrotron LX20 (Beckman, Palo Alto, CA). Immunoglobulin, including alpha1-globulin, alpha2-globulin, beta globulin and gamma globulin, was quantified by electrophoresis analyses using the Array™ Protein System (Beckman Instruments Inc. Brea, CA).

In both the spectrophotometry and electrophoresis analyses, the detection limits for the total protein, albumin, alpha1, alpha2, beta and gamma globulin were 30, 10, 0.1, 0.1, 0.1 and 0.4 g L⁻¹, respectively. The coefficients of variance (CV) (precise) for the total protein, albumin, alpha1-globulin, alpha2-globulin, beta globulin and gamma globulin were 5, 3, 5, 4, 5 and 5%, respectively, and they indicated an acceptable level of precision from an engineering point of view (less than 5%).

2.3. Confocal laser scanning microscopy measurement

Three different H₂S concentration serum samples were prepared, and they were called Serum sample 1, Serum sample 2 and Serum sample 3, respectively, in the following discussion. The serums were taken from 10–12 week old male Sprague-Dawley rats (rats were housed in an animal care facility at the College of Medicine, the University of Saskatchewan. Animal experiment protocols were approved by the Committee on the Animal Care and Supply of the University of Saskatchewan). The H₂S concentrations of the three samples, measured by an electrode, were 25, 44 and 71 μM, respectively. Each of these serum samples amounted to 0.2 ml.

The 0.5 mg CNTs were then treated with the three serum samples. In particular, the CNTs were put into the serum samples, taken out after 2 min, and dried for 30 min on glass slides for measurement.

The laser scanning microscope (Zeiss LSM 510 META) was used to measure the fluorescence of the CNTs which were treated by the serum samples with different H₂S concentrations (25, 44 and 71, respectively). The excitation wavelength selected in this experiment was 514 nm and emission was collected from 539 to 700 nm. Further in the experiment, for each sample, 30 different spots were measured, and each spot was measured 30 times (30 measurements of each spot took 1 min) by the time series function of the confocal laser scanning microscope.

2.4. Raman microscopy measurement

Hemoglobin was first mixed with fetal bovine serum from Sigma to scavenge H₂S; the serum sample after this treatment was considered 'without' H₂S. Then, this serum sample was equally divided into four parts (samples). Three of them had further H₂S added at different concentrations, and one sample was left as a control sample. The H₂S concentrations of the three serum samples were 20, 50 and 100 μM, respectively. Each of the three samples amounted to 0.2 ml.

The 0.5 mg CNTs were treated with the control serum sample and three other serum samples with different H₂S concentrations (20, 50 and 100 μM, respectively). In particular, the CNTs were put into the serum samples, taken

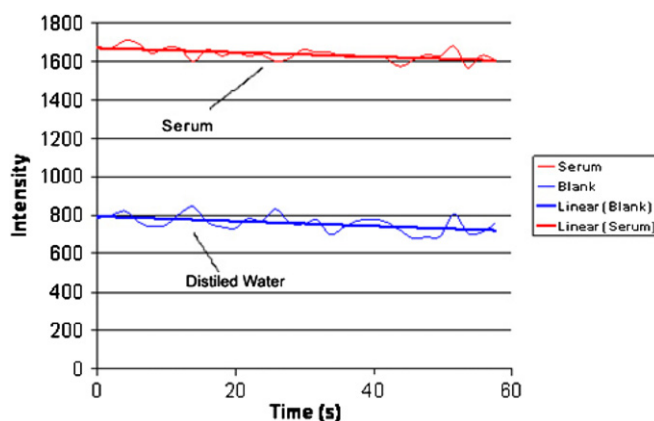


Figure 2. Fluorescence emission for the carbon nanotubes after immersion in the serum and distilled water. The x-axis is the measurement time and the y-axis is the intensity.

Table 1. Protein quantities before and after the serum was treated by carbon nanotubes (these results are from three measurements).

Sample	Control sample	Treated sample
Total protein (g L ⁻¹)	48	49
Albumin (g L ⁻¹)	16	17
Alpha1-globulin (g L ⁻¹)	3	3
Alpha2-globulin (g L ⁻¹)	8	8
Beta globulin (g L ⁻¹)	9	8
Gamma globulin (g L ⁻¹)	11	12

out after 2 min, and dried for 30 min on glass slides for measurement.

To enhance the validity of the result with the LSM fluorescence, Raman microscopy (Renishaw system 2000) was also used to measure the Raman luminescence of the CNTs which were treated with the four serum samples (one control serum sample and three other serum samples of different H₂S concentrations). In the Raman measurement, the excitation wavelength was 514 nm, and the emission wavelength of luminescence was chosen in the range from 520 to 600 nm.

3. Results and discussion

3.1. Serum protein measurement

The sulphide ion sensitive electrode shows that H₂S was present in the serum, and in particular the concentration of H₂S was about 36 μM. Spectrophotometry and electrophoresis analyses indicated that the amounts of proteins in the serum sample (control sample or not treated with the CNTs) and the serum samples treated with the CNTs were no different (see table 1), which suggests that the proteins were not attached to the CNTs.

3.2. Confocal laser scanning microscopy measurement

Figure 2 shows the fluorescence spectrum of confocal laser scanning microscopy for the CNT treated with distilled water and the CNT treated with the serum in which there were

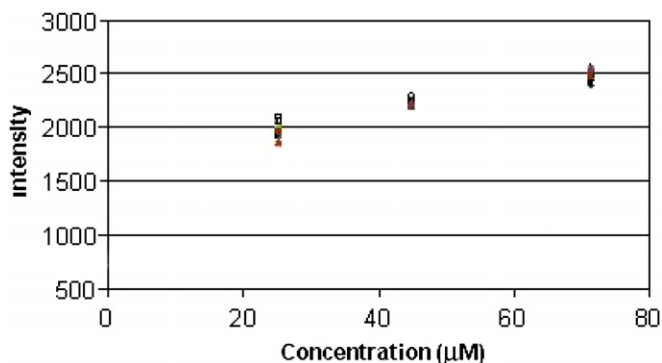


Figure 3. Fluorescence intensities for the carbon nanotubes after immersion in serum (25 μM , 44 μM and 71 μM , respectively). Thirty different spots were measured for each sample. Each spot was measured 30 times. Thirty points correspond to each concentration (25 μM , 44 μM and 71 μM , respectively). Each point was the average of 30 measurements.

both proteins and H_2S . The excitation wavelength selected in this experiment was 514 nm and emission was collected from 539 to 700 nm. From this figure, it can be seen that the intensity of the fluorescence emission for the CNT treated with the serum is higher than that for the CNT treated with distilled water. According to the result discussed in section 3.1, the higher intensity must not be caused by any protein in the serum but only by H_2S . Another support to this assertion is our previous work in which we showed that H_2S can bind with the unfunctionalized CNT and this binding can increase the intensity of the fluorescence emission of confocal laser microscopy from the CNT [17].

Figure 3 shows the fluorescence intensities by the confocal laser scanning microscopy of the CNTs treated with Serum sample 1, Serum sample 2 and Serum sample 3 that have 25, 44 and 71 μM H_2S concentration, respectively (see section 2.3). The excitation wavelength selected in this experiment was 514 nm and emission was collected from 539 to 700 nm. The variation bar for each sample was calculated based on the measurement on 30 spots on each sample. For each spot, the average of 30 measurements is plotted on the figure. From the figure, it can be seen that as the H_2S concentration increases, the intensity increases as well. This provides a general positive test to our hypothesis that the H_2S can bind with the CNT in the serum where some proteins are present.

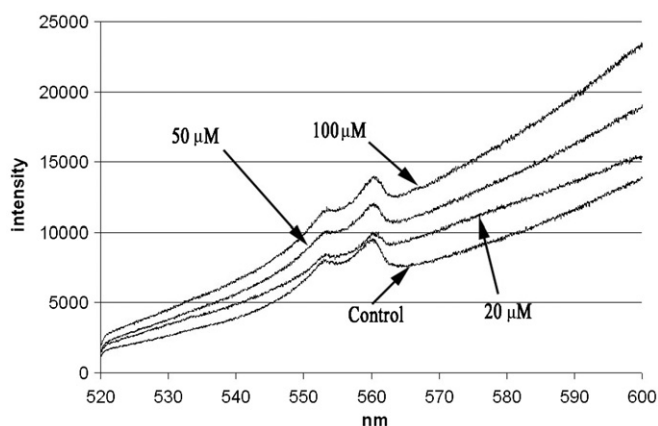


Figure 5. Luminescence of serum samples with different H_2S concentrations.

The regression and the residual analysis were further performed on the result of figure 3 to examine the relationship between the concentrations of H_2S and the fluorescence intensity. This analysis produced ANOVA (ANalysis Of VAriance between groups), line fit plot, residual plot and normal probability plot (figure 4). From ANOVA, we get $R = 3.95/4.24 = 0.93$ and $R^2 = 0.86$. It is known that if R^2 equals zero, there is no linear relationship between the dependent and independent variables; if R^2 equals 1, there is a perfect linear relationship. In our case, $R^2 = 0.86$ suggests that the relationship between the intensity and the H_2S concentration is quite linear.

The ‘standard error of estimate’, which is an estimate of the average spread of the residuals (or deviations) around the regression line, is represented by the square root of the ‘mean square (MS)’ associated with the residuals in the ANOVA table (i.e., square root of 3290 = 57.36). Note that its value is very similar in magnitude to the average standard deviations of the within-group variation in the original ANOVA and it has a similar interpretation—i.e., the average deviation of individual scores from the predicted group scores. Finally, the significance level associated with the F-test is less than 0.001, and thus we reject the null-hypothesis that the independent variable has no linear effect on the dependent variable.

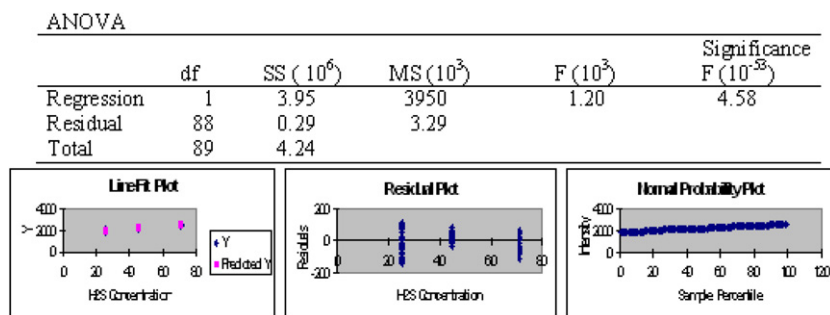


Figure 4. ANOVA, line fit plot, residual plot and normal probability.

From the line fit plot (figure 4), we can find that the relationship between the fluorescence intensity and the H₂S concentration is linear and satisfies the following equation:

$$y = 11.14x + 1707, \quad (1)$$

where y refers to the fluorescence intensity and x refers to the H₂S concentration. This linear relationship enhances our finding that H₂S can bind with the CNT in the serum where some proteins are present.

3.3. Raman luminescence measurement

The different intensities of the Raman luminescence of the CNTs which were treated with the serum samples of different H₂S concentrations (20, 50 and 100 μ M, respectively; see section 2.4) are shown in figure 5. From this figure, it can be seen that as the concentrations of H₂S increase, the intensities of the Raman luminescence also increase. This result is consistent with the result obtained from confocal laser scanning microscopy, which thus enhances the finding that H₂S can bind with the CNT in the serum where some proteins are present.

4. Conclusion with further discussion

This study concluded that (1) H₂S can bind with the unfunctionalized CNT in the serum where some proteins (e.g., albumin, etc) are present, (2) this binding can be well observed by the fluorescence of laser scanning microscopy and luminescence of Raman microscopy, (3) the intensity of the fluorescence of the unfunctionalized CNT treated with the serum of different H₂S concentrations is in a very good linear relationship with the H₂S concentrations, which further means that a sensor can be built, with the unfunctionalized CNT as a transducer and the LSM fluorescence as a signal acquisition modality, to measure H₂S in sera, (4) currently, the resolution is 20 μ M H₂S concentration in sera and the smallest quantity of H₂S is 0.5 μ g, and (5) the proteins in the serum do not bind with the unfunctionalized CNT under the instruments of spectrophotometry and electrophoresis.

The mechanism for the aforementioned conclusion (1) can be hypothesized. *First*, it is those defects on the unfunctionalized CNT that are attractive sites to attach H₂S. Defects have different mechanical and electrical properties; for example, the electronic density around defects is different from that in other parts, which can lead to different adsorptions at defects in comparison with the remainder of the surface lattice. Furthermore, the defects usually lead to the restoration of broken bonds by means of adsorption, so energy at the defect site is much larger than that at the regular site. It is noted that there seem to always be some defects on the unfunctionalized CNT, and these defects have such types as vacancies, dislocations and declinations and they are generated during the growth or by irradiation of CNTs [20].

Second, Rudee and Price [21] reported their experiment and concluded that human serum albumin (HSA) (molecular dimensions 8 nm \times 3.8 nm [22], with a monomolecular radius of gyration in pH 5–7 solution of 3.2–3.4 nm [23]) formed a

continuous film on an amorphous carbon surface in only 1.3 s of exposure. It is also reported that in the presence of activated carbon, H₂S can react with oxygen at low temperatures and produce sulfur and water [24, 25]. In our case, the CNT is very similar to activated carbon in terms of the phenomenon as observed and mentioned. Therefore, we hypothesize the following mechanism which governs that H₂S binds with the unfunctionalized CNT in the serum.

When the CNT is in the serum, the serum albumin formed a continuous film on the CNT surface. Although the CNT has the potential, especially at the defect sites, to adsorb the proteins in the serum, the large weight of proteins (usually in the kD range) makes them hard to move. For a small portion of proteins which can move toward the CNT, they are stopped by the serum albumin film. On the other hand, small molecules such as H₂S can easily move and pass through this film to the CNT. This process is simply physical adsorption where CNTs absorb H₂S by the force between molecules which is called van der Waals force. Before H₂S reaches the CNTs, the oxygen molecules have already been adsorbed on the CNT and have formed reactive points. The H₂S molecules that reach the CNT are partly dissociated into protons and hydrosulfide ions. The latter reacts with the oxygen at the reactive points to form hydroxyl ions and sulfur, and sulfur is adsorbed on the CNT. The protons neutralize the hydroxyl ions and produce water. The water is then desorbed from the surface, which is a chemical reaction step.

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