

A novel enzymatic method for determination of homocysteine using electrochemical hydrogen sulfide sensor

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1. ABSTRACT

Homocysteine is a sulfur-containing compound produced during metabolism process of methionine. Its uptake in human plasma is believed to be the cause of cardiovascular diseases and many other diseases. An electrochemical method was proposed for selective and quantitative measurement of homocysteine by employing hydrogen sulfide sensor coupled with methionine α , γ -lyase. The principle of this method is to measure the evolved hydrogen sulfide from the enzymatic reaction between homocysteine and methionine α , γ -lyase. The sensitivities of the measurements at different pH values of the tris buffer solutions and at room temperature peaked to 275 pA/ μ M at pH 6.5 with detection limit of 150 nM (based on 3 σ cutoff). The linearity measurements at pH 6.5 were performed for the homocysteine concentrations range from 0.5 to 200 μ M, which is wider than the human blood plasma total homocysteine level of 5 to 100 μ M, and the regressive analysis of the experiments gave $R^2=0.9987$. The enzyme also showed the fastest response to homocysteine in the tris buffer solution of pH 7.5 with the current approaching its maximum at 134 seconds. The interference tests against several common agents were carried out, and found that cysteine and methionine were the major two species to introduce measurement problem. The solution to this interference problem was explored and discussed thoroughly based on the preliminary tests. The sensitivities of the experiments against several enzyme concentrations were also performed.

2. INTRODUCTION

Since McCully first established the relationship of plasma homocysteine concentration with vascular disease in 1969 (1), hyperhomocyst(e)inemia caused by the elevated plasma total homocyst(e)ine level, i.e., the summation of free homocysteine, homocysteine thiolactone and covalently bonded homocysteines via a disulfide interaction, is now well recognized as one of the major culprits for cardiovascular diseases (2-8). The cause of building-up plasma homocysteine concentration is linked to the deficiencies of several vitamin cofactors, such as vitamin B6, folic acid (vitamin B7) and vitamin B12 (2). It was reported that homocysteine could make blood more likely to clot by increasing the stickiness of blood platelets and therefore, the heart attack or stroke would occur as the result of slowed and/or blocked blood flow. The subsequent studies on hyperhomocyst(e)inemia also linked this compound to many other diseases, such as Alzheimer's disease (9, 10), complications of pregnancy (11), birth defects (12), renal disease (13, 14), psychiatric disorders(15), cognitive impairment in the elderly(16) and bone fractures (17, 18). As a manner of dealing with the hyperhomocyst(e)inemia, the American Heart Association (AHA) has listed homocysteine as an independent risk factor of heart disease (19) and a routine examination of plasma homocysteine level was recommended (20). The total homocyst(e)ine concentrations in human plasma are in the range of a few μ M to hundreds of μ M, viz., 5 to 15 μ M for healthy people, 16 to 30 μ M for moderate

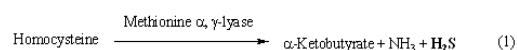
hyperhomocyst(e)inemia patients, 30 to 100 μM for intermediate and higher than 100 μM would be considered as very severe hyperhomocyst(e)inemia (21).

As a way to understand the role of homocysteine in relation to the above diseases, the demand on an accurate monitoring method of its plasma concentration among different patients becomes very high. The accuracy requirement immediately made the interference matter from similar amino acids, such as cysteine and methionine, an important issue because cysteine and methionine are structurally different from homocysteine by merely a $-\text{CH}_2$ group, and the recognition of homocysteine among them could be problematic. As these amino acids have considerably different pathologic meanings, the method to distinguish homocysteine from others is highly desirable.

There are many methods currently used for the determination of plasma total homocysteine. One of the commonly accepted measurement methodologies is to use the chromatographic technique to separate different species in plasma through a column and then use a unique detection method to obtain the homocysteine level, such as mass spectrum (22), fluorescence (23, 24) and electrochemistry (25, 26). However, these methodologies are time consuming as well as requiring sophisticated instruments with well-trained operators, and therefore, are not suitable for clinical applications. The immunoenzymatic method avoided the use of chromatographic column. But this method is relatively expensive due to the high cost of both reagents and equipment (27). A new optical method developed by Carolina Liquid Chemistries demonstrated reasonably consistent experimental results compared to the chromatographic technique and the immunoenzymatic method. However this method requires running three cumbersome enzymatic reactions before measuring the optical absorption and therefore, it can not be employed as a swift and economical technique (28). Electrophoreses coupled with either electrochemical (21) or fluorescence method (29) are still time consuming even though time consumption is shortened by the capillary technique. In addition, these methods also require expensive instruments and therefore, are not very applicable. The homocysteine test kit utilizing the recombinant homocysteine α , γ -lyase (30) coupled with fluorescence measurement method uses the indirect way to detect to hydrogen sulfide released from the enzymatic reaction of homocysteine (31, 32). But this method illustrated significant interferences from cysteine, methionine as well as glutathione and therefore, may cause some false measurements. The attempt to achieve the interference reduction of this kit requires performing the test within a specific time, i.e., 3 minutes or so, in order to let the test complete before the slower interfering enzymatic reactions start. Unfortunately, the rate of enzymatic reaction is not only dependent upon the species in the system but is also very closely related to the testing environment such as temperature, amount of enzyme and many other factors. Therefore, to control the specific timing in order to reduce the experimental uncertainties becomes impossible. The electrochemical method using nano-technology exhibited an enhanced sensitivity towards to homocysteine (33) but it is only applicable for post-

separation purpose, such as coupling with chromatographic method. The first bio-homocysteine sensor using immobilized D-amino acid oxidase showed a very poor selectivity and low sensitivity towards to homocysteine (34) and the chance for this kind of biosensor to become a clinical testing apparatus is also ruled out.

This study will focus on utilizing an electrochemical hydrogen sulfide sensor with a very active enzyme, i.e., methionine α , γ -lyase, to carry out the swift test for determining aqueous homocysteine concentration. The principle is that the enzymatic reaction of homocysteine and methionine α , γ -lyase would produce hydrogen sulfide stoichiometrically. This enables the indirect determination of homocysteine possible via determining the concentration of released hydrogen sulfide. The principle is depicted in equation 1 as follows:



Subsequent experiments verified that the reaction is indeed stoichiometric for free homocysteine as long as the appropriate amount of enzyme is used.

3. EXPERIMENTS

3.1. Chemicals and reagents

Methionine α , γ -lyase was obtained from Formosa Biomedical Technology Corporation (Taiwan) in a way that a plate-like rack holds 96 (8x12) small wells and each well equally contains 2.5 μg dry enzyme. The volume of each well is slightly greater than 200 μL and enzyme was cold stored (-20°C for long term and -4°C for short term) within the well until needed. Each well is easy to break and separate from others for individual experiments. The enzyme was directly dissolved into buffer solution prior to each experiment without further treatment.

DL-homocysteine, L-cysteine, L-methionine, dopamine, L-ascorbic acid, Trizma® hydrochloride, ammonia, hydrochloride, potassium hydroxide, potassium nitrite, and hydrogen peroxide were purchased from Sigma or Aldrich (St. Louis, MO, USA) and were used without further purification. DL-homocysteine, L-cysteine and L-methionine solutions were made in 10 times (10x) higher than their experimental concentrations and stabilized by ascorbic acid at the molar ratio of at least 1 to 1. These solutions were stored in a freezer until needed. Other solutions were prepared and were either frozen or refrigerated during the storage.

Tris buffer solutions at different pH values were prepared by making 0.1 M Trizma® hydrochloride solutions followed by adjustments of using either 0.1 M potassium hydroxide or 0.1 M hydrochloride solution to desired pH while monitored by a Jenco pH meter.

3.2. Instrumentation

The measurements were carried out using Apollo 4000 Free Radical Analyzer (World Precision Instruments, Sarasota) and WPI ISO-H2S-2 hydrogen sulfide sensor

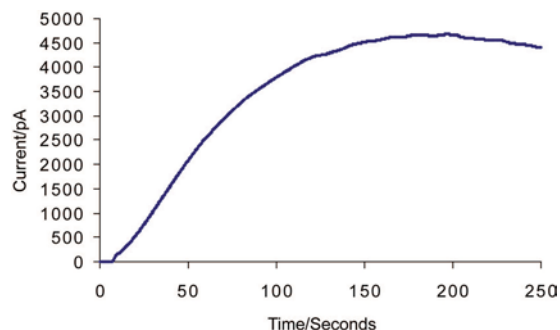


Figure 1. A typical measurement of 10 μM homocysteine at pH 6.5 by Apollo 4000. The current maximized at about 194 seconds to 4678 pA.

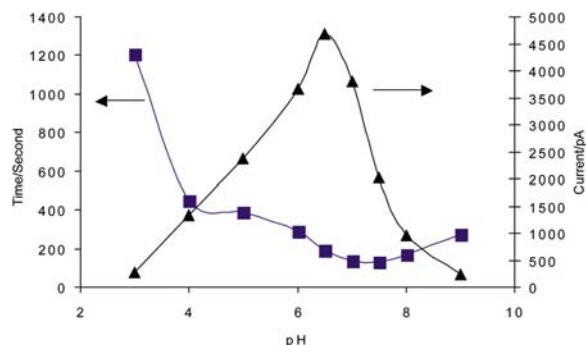


Figure 2. Response of 10 μM homocysteine at different pH tris buffer solutions (triangle) and their relative response times (square).

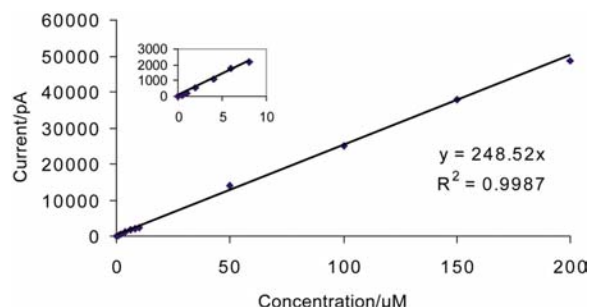


Figure 3. The responses of homocysteine to the ISO-H2S-2 sensor from 0.5 μM to 200 μM . Inset: the low concentration of homocysteine from 0.5 μM to 8 μM .

(World Precision Instruments, Sarasota). The sensor was operated at bias potential of 150 mV. Deionized water was generated by running commercially available distilled water through the Millipore Simpak 1 filter. The resistivity of the de-ionized water was 18.2 Mohm.cm. pH meter is from Jenco Electronics, LTD, Model-671P.

3.3. Experiments

Experiments were carried out by pipetting 180 μL of an appropriate buffer solution into the dry enzyme well with a micro-stir bar. The tip of WPI ISO-H2S-2 hydrogen sulfide sensor was immersed into the enzyme-buffer solution while stirring. An injection of 20 μL of an appropriate testing solution (10x) will be made after the amperometric current stabilized. Figure 1 revealed a

typical measurement of 10 μM homocysteine at pH 6.5. The amperometric current peaked to 4678 at 194 seconds.

4. RESULTS AND DISCUSSION

All the measurements were repeated at least three times in order to obtain the desired repeatability. The experiments of pH effect, sensitivity, linearity, selectivity against several common reagents and enzyme concentrations were carried out and will be discussed in this section.

4.1. pH effect

The experiments of the WPI ISO-H2S-2 hydrogen sulfide sensor responding to the hydrogen sulfide that was generated from the enzymatic reaction of homocysteine (equation 1) were carried out at pH values ranging from 3.0 to 9.0. The homocysteine concentrations of each measurement were kept at 10 μM and amount of enzyme used was 2.5 μg . It was found that the response peaked at around pH 6.5 as depicted in Figure 2. It is obvious that the enzyme demonstrated the highest current response at this pH value and therefore, pH 6.5 was used for the subsequent experiments.

Investigating the pH dependence graph, it is noticeable that the change toward the acidic side (low pH value) of the peak (pH 6.5) is relatively smoother than the basic side (high pH value). The enzymatic reaction in an acidic environment is relatively slow. The test at pH 3.0 can be as slow as over one thousand seconds before it reaches its amperometric peak. A slight increase of the pH could dramatically speed up the reaction rate to only a couple of hundred seconds. The reaction rate was maximized at pH 7.5 for the current to reach the peak in only 134 seconds. The results of reaction rates corresponding to pH values were also depicted in figure 2 and tabulated in Table 1.

4.2. Sensitivity and linearity

The experiments with different concentration of homocysteine ranging from 0.5 μM to 200 μM were performed at pH 6.5. Figure 3 showed the experimental data along with the regressive analysis results. As indicated in figure 3, it is evident that the WPI ISO-H2S-2 sensor performed with excellent linearity ($R^2=0.9987$) on the measurements in a concentration range of total homocysteine wider than the actual human physiological concentration. This ensured for its suitability for clinical usage. In addition, the detection limit for this method can be as low as about 150 nM (3σ cutoff), which is far sensitive than the current homocysteine test kit available commercially (detection limit 1 μM) (31, 32). This great enhancement of detection limit not only rendered the ISO-H2S-2 sensor method to measure homocysteine in a more accurate manner but also created way to detect different forms of homocysteine in the blood plasma. In other words, this method may eventually render the ability to distinguish the homocysteine in the free form and bonded form from the mixture of total plasma homocysteine because the methionine α,γ -lyase only reacts with the free form (reduced form) of homocysteine actively. As the

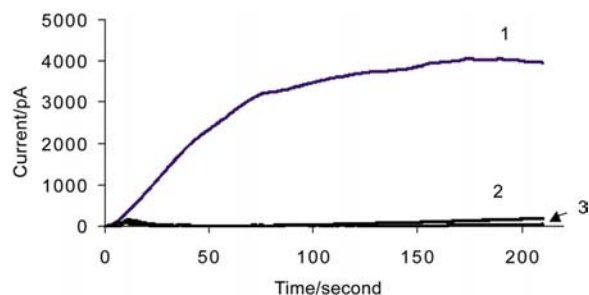
Table 1. Summarize the pH dependent experiments of the homocysteine enzymatic reaction at 10 μM

pH	3	4	5	6	6.5	7	7.5	8	9
Response Time at Peak (Second)	1209	448	388	289	194	136	134	168	272
Response Current (pA)	267	1338	2367	3668	4678	3804	2010	970	231

Table 2. Interference tests of WPI ISO-H2S-2 sensor against certain compounds

Compounds	NH_3	H_2O_2	KNO_2	Dopamine ¹	Ascorbic ¹ Acid	GSH ¹
Concentration (μM)	60	8	80	20	1000	100
% Interference	0	0	0	0	0	0.27

¹ Tests were under the same condition of homocysteine tests, i.e., with tris buffer pH 6.5 and methionine α , γ -lyase.


Figure 4. The responses of same concentration of homocysteine (1), cysteine (2) and methionine (3).

usual level of the free unbound homocysteine species is from only hundreds nM range (35) for healthy people to an elevated level for cardiovascular patients (36), the monitoring of free homocysteine as well as bound homocysteine could be crucial to the medical community as a cardiac marker. The study of the cause of cardiovascular disease can be better divided to associate to different forms of homocysteine at different extents when the highly sensitive detection technique is available. Therefore, this electrochemical detection method could possibly become the first candidate with the capability to fulfill this requirement as there is no other method yet possessing such a high sensitivity for the measurement of low homocysteine concentrations.

4.3. Interference

Selectivity was tested against several possible interferers such as ammonia (NH_3), hydrogen peroxide (H_2O_2), potassium nitrite (KNO_2), dopamine, ascorbic acid and glutathione (GSH). Table 2 listed the results of the measurements.

Basically, most of the compounds listed in Table 2 did not cause any interference except glutathione, which exerted only a small amount of interference at relatively high concentration. Consequently, the interference from these compounds would be insignificant in this electrochemical method especially when the interference reduction by real time dynamic technique (discussed later) was employed. The more severe interference stems from cysteine and methionine, and more attention was paid to them during the interference studies.

As an experimental method to study the individual profile of each species, all the interference tests and homocysteine test were performed individually and the

interference calculations were made by combining the scaled digital experimental data together into the same graph. The experiments on mixtures of relative binary systems, such as the mixture of homocysteine and cysteine at the specific molar ratios, were also made, and the similar interference results were observed.

4.3.1. Interferences of cysteine and methionine at equal molar concentration

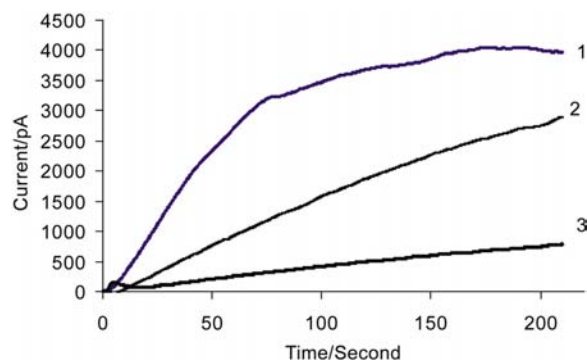
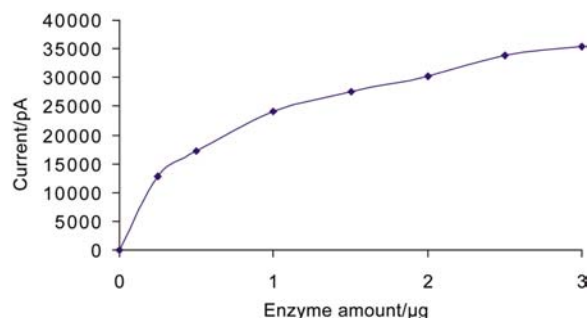
Figure 4 displayed the amperometric current profiles of 10 μM homocysteine, cysteine and methionine in tris buffer at pH 6.5. Examining this diagram, the homocysteine current apparently peaked at 175 seconds. At this peak point, the interferences, i.e., the current contributions from cysteine and methionine at 175 seconds, were relatively small at 3.71% and 0.15% respectively. This is because the enzyme activities for cysteine and methionine are both much slower than homocysteine and they sluggishly peaked at a much later time. This selectivity feature of the enzymatic reactivity would allow us to distinguish the homocysteine from cysteine and methionine by choosing an early reaction time when the majority of current is contributed from homocysteine enzymatic reaction, and consequently, the interference can be greatly reduced. This real time dynamic technique is actually the same methodological strategy used by homocysteine test kit (31, 32) but our method is superior over the test kit because WPI Apollo 4000 system has much better control on monitoring the timing of the reaction process plus the more active enzyme can be used.

It is noticed that there is a small discrepancy on the peak response time of this experiment (175 seconds) from pH dependent test (194 seconds). This discrepancy might come from the use of different sensors in these experiments and the slight changes of environmental factors, such as the ambient temperature, injection location relative to sensor tip, injection speed, possible manually induced agitation and others.

Examining the details of the current profiles, there is a current spike at beginning of the experiments for several tests. That seems to relate to the sample injections as the experiments were performed in such a small vessel that the injection-associated high local transient concentration gradient at around the sensor tip along with mechanical agitation might be the cause the spike. Fortunately, the spike lasted not too long and the useful parts of data were all falling into much later time. Consequently, this current spiking would not cause any problem to our study.

Table 3. Interferences of homocysteine measurements from cysteine and methionine at different data acquisition time

Time (second)	Hcy peak %	Cys. Interference %	Met interference %
175	100	62.18	15.98
78	80	37.46	9.15
42	50	30.26	6.78
30	35	27.84	5.89

**Figure 5.** Response of 10 μM homocysteine (1) compared to 10 times higher of cysteine (2) and methionine (3).**Figure 6.** The experiments of response current (pA) against the weight (μg) of enzyme in 200 μl solution.

4.3.2. Interference of cysteine and methionine at 10 times higher concentration

The purpose of these tests was an attempt to bring the interference issue close to the physiological level of cysteine and methionine in human blood plasma, where the cysteine is around 250 to 274 μM for middle range (37), and methionine can be from 30 up to about 100 μM (38).

The Specimens containing either 100 μM of cysteine or methionine were performed and the results are shown in Figure 5. From this figure, it can be noticed that the interference from both cysteine and methionine became obvious. This is probably due to the enzymatic activity for low concentrations being so sluggish that the reactions of both cysteine and methionine were further delayed. The specific interferences at several data acquisition times are listed in Table 3. It is clear that the enzymatic activity of methionine α , γ -lyase for homocysteine is higher (faster) than cysteine and methionine. Once again, this feature rendered a way to reduce the inference of homocysteine measurements by cutting the data acquisition even before the homocysteine reaction peaked.

Nevertheless, the interference from methionine can be reduced to an accepted level with our real-time dynamical electrochemical method using WPI Apollo 4000 Free Radical Analyzer System and WPI ISO-H2S-2 hydrogen sulfide electrochemical sensor. This combination would be superior over other methods as previously described such as the homocysteine assay kit (31, 32), in which the key requirement is to use appropriate amount of enzyme and most importantly to have the right timing to run the experiments. In that method, the less active enzyme, i.e., homocysteine α , γ -lyase has to be used as the fast enzymatic rate of methionine α , γ -lyase may cause the measurement within correct timing impossible. In addition, the use of homocysteine α , γ -lyase to detect homocysteine also suffered from the cysteine interference problem and the concentration of cysteine used in that work was not reported (31, 32).

The relatively high interference from cysteine was successfully reduced to lower than 30% in these preliminary tests, but this is still not an acceptable level. The physiological cysteine level can be a few times higher than this experiment and the actual interference could be even higher. It is noticed that the interference tests in this work were under the pH value of the most methionine α , γ -lyase enzymatic activity condition towards to homocysteine responses, i.e., pH 6.5 (tris buffer). This may not be the best condition for minimizing interference. From the reaction rate point of view, the enzymatic reaction for homocysteine was actually maximized at pH 7.5 and that might be a better pH environment even though the actual response (to homocysteine) would be reduced slightly. Future work will aim at optimizing the best conditions in order to substantially reduce or completely remove the interference problem. That could involve fine-tuning the pH buffer solutions to minimize the interference and/or using the differences from the adducts' stability of homocysteine and cysteine with aldehydes for in situ separation of these two species (39, 40) in order to reach the goal of low or no interference from cysteine. The use of another enzyme to quench the cysteine in solution, such as cysteine dioxygenase (41), might be useful for pretreatment of the sample for cleaning up the interference. Either way, there are many methods that can be used to solve the interference problem.

4.4. Effect of enzyme concentration

The responses against different enzyme concentrations were also tested from 0.25 $\mu\text{g}/200\mu\text{l}$ to 3.0 $\mu\text{g}/200\mu\text{l}$. The results are depicted in figure 6. It seemed that the enzymatic reaction started to become saturated from 1.5 $\mu\text{g}/200\mu\text{l}$. This was also confirmed from the fact that at 1.5 $\mu\text{g}/200\mu\text{l}$ the response time started to decrease substantially to below 3 minutes as shown in figure 7. Consequently, our experiments were all under saturated enzyme concentrations.

4.5. Repeatability

Five separate measurements were made on homocysteine experiments at concentration of 10 μM and

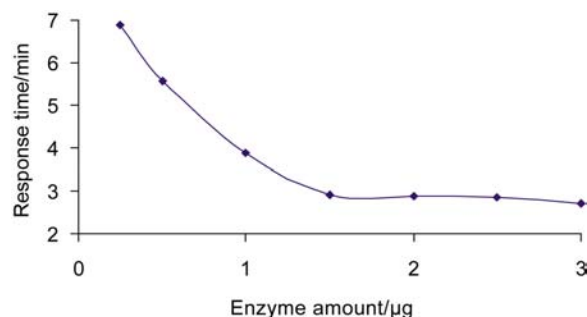


Figure 7. The relationship of response time (minute) and enzyme amount (μg).

100 μM. The errors for these measurements were reasonably low at 1.88% and 1.48% respectively. The sensor exerted an excellent repeatability among the measurements and, once again, demonstrated the superior of the electrochemical method.

4.6. Stability of homocysteine, cysteine and methionine

Homocysteine, cysteine and methionine in solutions are easily turned denatured even in freezer. Their solutions at room temperature or in refrigerator (-4 °C) were liable to lose the activities within a short period of time. This caused the inconsistent experimental data even though the solutions were freshly prepared daily. The stability tests were carried out with the solutions containing certain amount of ascorbic acid and being kept in refrigerator (about -4°C) until needed. The control experiments showed no significant change of the current response of the solutions in the enzymatic reactions for a span of 12 days. The anti-oxidant property of ascorbic acid should contribute to this enhanced stability of solutions. As described previously, ascorbic acid did not cause any interference problem in the homocysteine measurement, and thus all of the solutions in our experiments were then pre-made under the protection of ascorbic acid and kept in freezer (about -20°C) until needed. The solution remains stable for months under this condition.

5. CONCLUSION

The novel method using WPI Apollo 4000 Free Radical Analyzer system and WPI ISO-H2S-2 hydrogen sulfide sensor coupled with using methionine α, γ-lyase offers a great improvement to measure homocysteine over other methods in an accurate, fast, highly sensitive and timely manner. The detection limit of this method is enhanced several times compared to other's work. This low detection limit may create a new way to detect different forms of plasma homocysteine. The linearity is high at $R^2 = 0.9987$ for a wider range of homocysteine level than human plasma. This powerful system utilized a real-time dynamic data acquisition method and solved the timing problem that resided in other measurement methods. The success of this work along with future solutions of complete elimination of cysteine interference will offer the basis for the possibility of building up the most sophisticated homocysteine biosensor. To the best of our knowledge, it is the first attempt to use the indirect

enzymatic electrochemical method by sensing hydrogen sulfide to quantitatively detect the aqueous homocysteine level.

6. ACKNOWLEDGMENT

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