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A novel glucose chemiluminescence biosensor based on a rhodanine derivative chemiluminescence system and multilayer-enzyme membrane

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Using glucose oxidase as a model enzyme, a novel rhodanine derivative chemiluminescence biosensor for the determination of glucose was formed based on multilayer-enzyme membrane as receptor, which was assembled via layer-by-layer assembly of sol-gel and glucose oxidase-gold nano-particles inside a glass tube. Compared with the traditional chemiluminescence biosensor, the proposed biosensor had some remarkable advantages, such as good selectivity of substrate, good response performance, good stability, good sensitivity, and longer service life. The linear range was from 1.0×10^{-6} to 5.0×10^{-2} mol L^{-1} ($r = 0.9991$). The detection limit was 5.0×10^{-7} mol L^{-1}. In this work, the optimum number of layers was 4, and the Michaelis-Menten constant of immobilized glucose oxidase was 0.2 mmol L^{-1}. The proposed method was applied to the determination of glucose in 6 different human serum samples with satisfactory results.

Key Words: Rhodanine derivative, chemiluminescence, enzyme, layer-by-layer, sol-gel

Introduction

Due to its importance in many fields,[1,2] the detection of glucose has been the subject of considerable efforts. For example, clinical diabetes monitoring has been mainly based on blood glucose measurements.[2] In recent years, a number of studies have been conducted to develop new glucose detection methods.[3–6] The flow injection-chemiluminescence (FI-CL) biosensor is most popular, mainly involving the traditional catalyzed-luminol oxidation[7] wherein light emission occurs instantaneously upon merging the oxidant resulting from a

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previous enzymatic substrate transformation in the heterogeneous phase with the chemiluminescence reagent and the catalyst. Typically, the enzymatic reaction between glucose and glucose oxidase (GOD) produced gluconic acid and hydrogen peroxide (H_2O_2). Then the determination of glucose can be achieved via CL reaction between the enzymatic produced H_2O_2 and luminal; this reaction can be catalyzed by horseradish peroxidase (HRP).[4] However, besides its sensitivity, it suffers from some disadvantages such as complicated experimental setup and an extra analysis cost of HRP, which is very expensive, and the introduction of HRP will result in a new unstable factor.

To the best of our knowledge, no reports about determination of glucose by rhodanine CL system have been published to date. In this work, for the first time, a novel rhodanine derivative (3-(2′-nitryl)-5-(4′-methyl-2′-sulfonicphenylazo) rhodanine) was synthesized by substituting the -SO_3H group, which has great complexing capability to rhodanine, whose CL properties were found to be superior. Its reaction with acid potassium permanganate could be catalyzed strongly in the presence of H_2O_2; hence, in our recent work it was used in a CL system to replace traditional luminal-HRP. It was found that the CL intensity of the system could be enhanced remarkably in the presence of H_2O_2 to obtain higher sensitivity, better selectivity, and shorter response time; thus the goals of simplifying the experimental setup and reducing the analysis cost could be achieved.

The layer-by-layer assembly (LBL) method is one of the most perspective methods for thin film deposition.[8] This technique is based on the alternate electrostatic adsorption of the negatively/positively charged individual components. This new deposition technique paves the way to fabricate sensing membranes with some advantages such as controlled thickness, structural morphology, and biocatalyst loading. It has been successfully applied to the preparation of thin films of various proteins and nanoparticles.[9−11] In this work, a novel multilayer-enzyme membrane was assembled via layer-by-layer assembly of sol-gel and glucose oxidase (GOD)-gold nano-particles (AuNPs) inside a glass tube. Enzymes adsorbed on AuNPs retain their activity because AuNPs provides a surrounding similar to the native surrounding of redox proteins.[12] The sequential repetition of the deposition of sol-gel and enzyme-AuNPs could produce a multilayer membrane with a porous structure, which would be favorable for the approach of substrates to the enzyme molecules in internal layers. The novel multilayer-enzyme membrane, which avoided the drawbacks of conventional methods such as short service life and high detection limit, had high selectivity, binding activity, and reproducibility simultaneously.

Experimental

Synthesis of novel rhodanine derivative

The novel rhodanine derivative (3-(2′-nitryl)-5-(4′-methyl-2′-sulfonicphenylazo) rhodanine) (M2NRASP) had been synthesized for the first time by the author.

Into a 250 mL conical flask were placed 30 mL of ammonia (China National Medicines Corporation Ltd.) and 10 mL of carbon disulfide (China National Medicines Corporation Ltd.), and 13.8 g of o-nitroaniline (China National Medicines Corporation Ltd.) was added later while stirring. The above mixture was left to stir for 24 h and then pumping filtrated and washed with ether (Tianjin SHENTAI Chemical Industry Co., Ltd.), and the dithio-o-nitroaniline amine formate ammonium was obtained as (a). Then 18.8 g of chloroactic acid was neutralized to pH 7.0 with 8.0 g of sodium hydroxide in 20 mL of water. Afterward (a) was put in the neutralized

solution, and 65 mL of concentrated hydrochloric acid was added, which was heated to boiling and at once was filtrated; then a straw yellow solid was obtained, which was called 3-o-nitrobenzene rhodanine (b).

The diazonium salt was obtained by mixing 0.01 mol of p-toluidine arsenate (The British Drug House Ltd.) with 8.0 mL of hydrochloric acid (V_{HCl}:V_{H20} = 1:1), and adding 0.01 mol of sodium nitrite to the mixture in an ice bath.

In the ice bath, 0.01 mol of (b) was added to 10 mL of ammonia; afterwards the diazonium salt was injected, making sure that the pH of the solution was about 8-9. After 1 h of stirring, the mixture was filtrated, and the filtrate was acidized with concentrated hydrochloric acid (at room temperature), and precipitate was found. The M2NRASP was obtained by filtrating and drying. The synthetic route is shown in Figure 1.

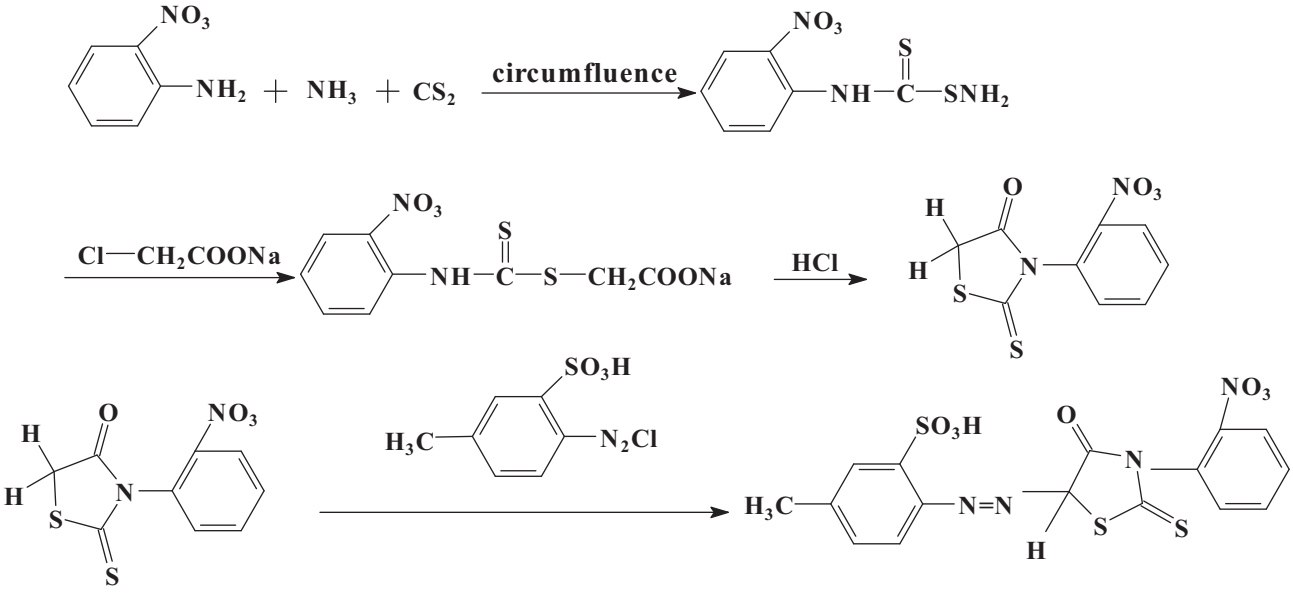

Figure 1. The synthetic route of M2NRASP.

Data from the infra-red spectrogram (*KCl discs*, cm^{-1}) are shown as follows:

υ_{OH} = 3446; $\upsilon_{N=N}$ = 1456; $\upsilon_{C=S}$ = 1265; υ_{Ar-C-N} = 1265; $\upsilon_{o,p,p-substituted}$ = 826; $\upsilon_{S=O}$ = 1492; $\upsilon_{benzenering\ \ (C=C)}$ = 1549, 1629, 1498; υ_{C-N} = 1022; υ_{NO2} = 1386.

The elementary analysis of M2NRASP showed a composition of C (41.76%) H (2.53%) N (11.92%), which is in good agreement with the theoretical composition of M2NRASP: C (42.47%) H (2.67%) N (12.38%).

Apparatus and manifold

The IFFM-E flow injection chemiluminescence analyzer (Xi'an Remex Electronic Instrument High-Tech Ltd. Xi'an, China) was hooked up with an automatic injection system and a detection system. A polytetrafluoroethylene (PTFE) tube (0.8 mm i.d.) was used to connect all of the components in the flow system. The CL intensity was analyzed with a personal computer. Double-distilled water was obtained by SYZ-550 quartz sub-boil high-purified water distiller (Jiang Su Jin Tan, Jiang Su, China). Weighing of all chemicals was carried out using

a Mettler-toledo analytical balance (Shanghai, China). The pH measurements were made using a home-made PHS-3C digital pH-meter (Shang Hai Lei Ci Device Works, Shanghai, China) with a combined glass-calomel electrode. The elementary analysis was carried out with an elementary analysis meter (Model 2400II CHNS/O, Perkin-Elmer, USA). An infrared spectrum meter (Nicolet 380, Thermo Electron Corporation, USA) was used to attribute chemicals.

All reagents were of analytical reagent grade or above. A 1.0×10^{-2} mol L^{-1} stock glucose-PBS standard solution was obtained by dissolving accurately 0.9008 g of glucose (Aladdin, Shang Hai, China) with a little doubly distilled demineralized water and then diluting to 250 mL accurately with phosphate buffer solution (PBS) (pH 7.4). Working glucose-PBS standard solutions was prepared by dilution of this stock standard solution with PBS. A stock standard solution containing 1.0×10^{-4} mol L^{-1} M2NRASP was prepared by dissolving 0.2260 g of M2NRASP with anhydrous alcohol and then diluting to 250 mL accurately with anhydrous alcohol. A 2.0×10^{-5} mol L^{-1} working standard solution of M2NRASP was obtained by dilution of the stock standard solution with anhydrous alcohol. A 5.0×10^{-3} mol L^{-1} stock solution of potassium permanganate was prepared by dissolving 0.0790 g of $KMnO_4$ (Aladdin, Shang Hai, China) with 500 mL of water.

Fabrication of multilayer-enzyme membrane receptor

The sol-gel stock solution was prepared following the proposed method.[14] Ethyl orthosilicate, H_2O, and HCl (0.1 mol L^{-1}) were mixed in a glass vial under content ratio. The mixture was sonicated for 1 h and then a clear sol-gel stock solution was obtained. The stock solution was stored in a refrigerator at 4 °C. The pH of the stock solution was adjusted to 6.5 when it was used.

Then 0.5 g GOD was dissolved in 1 mL of PBS. The AuNPs stock solution was prepared following the proposed method.[13] Briefly, a sodium citrate solution (1%, 4.0 mL) was rapidly added to a boiled $HAuCl_4$ solution under vigorous stirring. The mixed solution was boiled for 10 min, and further stirred for 15 min. The resulting solution was cooled to room temperature and filtered, and then stored in the refrigerator (4 °C) ready for use. Then GOD-AuNPs solution was obtained by mixing GOD solution with 3 mL of AuNPs solution. The self-assembly layer-by-layer process was as follows: (1): 0.5 mL of sol-gel stock solution was coated on the inside of a straight glass tube (2.5 mm i.d. × 10 cm length) with ceaseless circumrotation for 10 min; then polymerization occurred and a gel was formed at room temperature after 10 min. (2): Using the same procedure, a layer of GOD-AuNPs was coated slowly to prevent loss of the sol-gel membrane. Thus the monolayer-enzyme membrane was obtained inside the glass tube. (3): Setup (1) and (2) was carried out 4 times to prepare a multilayer-enzyme membrane. Finally, the glucose-sensitive receptor was stored in a refrigerator at 4 °C for 12 h.

Procedure

As shown in Figure 2, 3 PTFE tubes (a, b, c), which were pumped by the main pump, were inserted into the M2NRASP solution, potassium permanganate solution, and HCl, respectively. One PTFE tube (d), which was pumped by the deputy pump, was inserted into the sample solution/glucose-PBS standard solution or PBS (blank solution). The main pump was started first to wash the whole system until a stable baseline was recorded. Then the deputy pump was started to inject the sample solution/glucose-PBS standard solution and PBS to

obtain the CL intensity of sample/glucose-PBS standard solution (I) and that of PBS (I_0) respectively. The concentration of glucose was quantified via the relative CL intensity (ΔI), which was obtained by subtracting the I_0 from I.

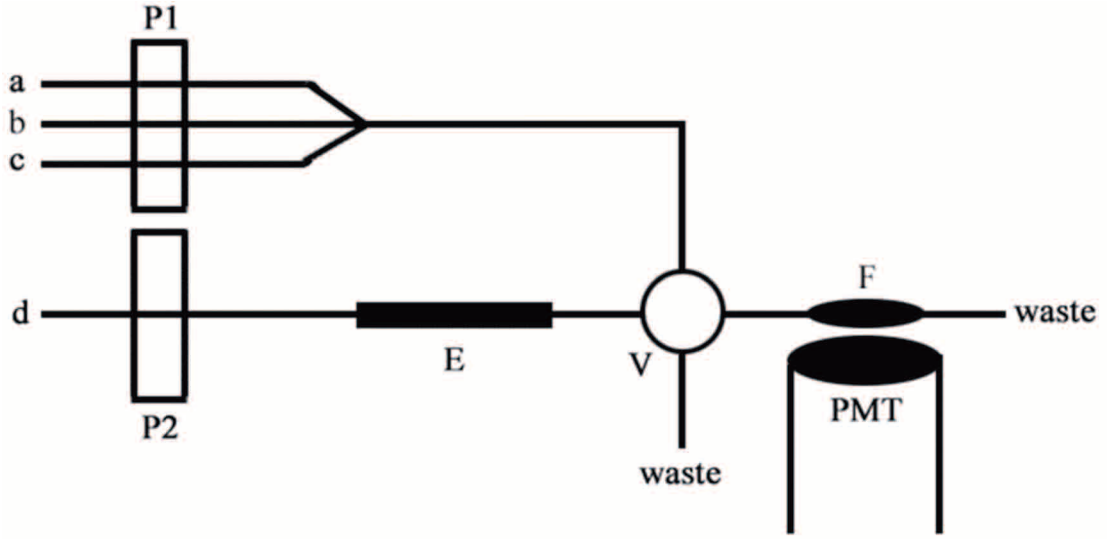

Figure 2. Schematic diagram of the CL biosensor for the determination of glucose a- potassium permanganate b- M2NRASP c- HCld- sample solution (or glucose-PBS standard solution or PBS blank solution) P1- main pump P2- deputy pump V- 6-way-valve E- glucose-sensitive receptor F- hover flow cell.

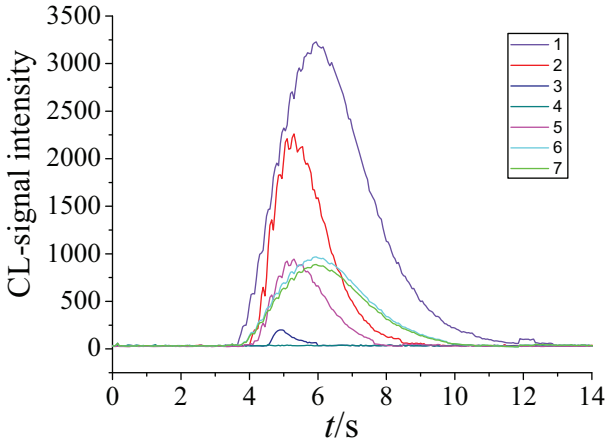

Figure 3. The CL kinetic curves of the system 1- HCl + $KMnO_4$ 2- HCl + $KMnO_4$ + H_2O_2 (GOD + glucose) (5.0×10^{-5} mol L^{-1}) 3- M2NRASP + HCl + $KMnO_4$ 4- M2NRASP + HCl + $KMnO_4$ + glucose (5.0×10^{-5} mol L^{-1})5- M2NRASP + HCl + $KMnO_4$ + GOD + glucose (5.0×10^{-5} mol L^{-1})6- M2NRASP + HCl + $KMnO_4$ + GOD-AuNPs + glucose (5.0×10^{-5} mol L^{-1})7- M2NRASP + HCl + $KMnO_4$ + GOD-AuNPs + glucose (1.0×10^{-4} mol L^{-1})

Results and discussion

Kinetic characteristics of the CL reactions

The CL intensities of all systems were detected, as shown in Figure 3. Any one of acid potassium permanganate, M2NRASP, and H_2O_2 is indispensable to obtain strong CL intensity (curves 1, 2, 3, 4, and 5). Once GOD was replaced by GOD-AuNPs, the CL intensity increased remarkably (curve 6 and 7) as the concentration of glucose solution increased. Hence, the $KMnO_4$-HCl-M2NRASP-H_2O_2 CL system could be used to determine glucose quantitatively.

The optimization of glucose-sensitive receptor variables

The number of layers is a key factor in this multilayer-enzyme membrane biosensor, and it has a great influence on service life directly and CL intensity indirectly. It was investigated under the same receptor size (2.5 mm i.d. $\times$ 10 cm length) from 2 to 6 layers. The experimental results showed that the CL intensity firstly increased with increasing number of layers, probably it was because the amount of the absorbed enzymes increased. However, above 4 layers, the CL intensity declined probably because the thickness of multilayer-enzyme membrane was too thick that the surface of the membrane started breaking off. Therefore, the number of layers of 4 was chosen for further study.

The receptor size (length and inner diameter (i.d.)) could influence the space and time of the enzymatic reaction and it also has a great influence on service life directly and CL intensity indirectly. With the length of receptor at the range of 6.0 to 13.0 cm, the CL intensity increased firstly with increasing length of receptor from 6 cm, probably because the enzymatic reaction time increased. However, the produced H_2O_2 could decrease because of the disproportionation; hence, the CL intensity started declining due to the decrease in H_2O_2 when the length of receptor was longer than 10 cm. Therefore, the length of receptor of 10 cm was chosen as optimum. Figure 4 shows that the service life of the receptor increased with increasing i.d. of receptor, which was due to the washing action of fluid decreasing. However, the detection limit of the biosensor increased with increasing i.d. of receptor, probably because the unreacted glucose increased. Considering the requirement of practical application, the i.d. of receptor of 2.5 mm was chosen as optimum. Under the optimum condition stated above, the reusability and storage stabilities of this biosensor were confirmed through repeated use and enzyme maintaining activity. The intensity of the CL signal was stable (R.S.D.% $<$ 5%) when the successive repeated time of the biosensor was less than 350. When the successive repeated time of the biosensor was more than 500, a 30% decrease in the intensity of the CL signal was observed. The intensity of the CL signal was measured as a function of storage time at room temperature. After a 4 week period immobilized GOD had lost only 30% of enzyme activity, whereas free GOD lost all activity. The high CL intensity of this biosensor, which indicated the high activity of the immobilized GOD, makes it a more suitable preparation for a variety of applications than traditional work.[4]

Determination of the Michaelis-Menten constant of GOD

The Michaelis-Menten constant (K_M) of GOD, which is a characteristic constant, depends on the category of enzyme and the concentration of enzyme does not influence it. The higher K_M values are, the weaker affinity

to substrate is and vice versa. The Michaelis-Menten equation is transferred to the following form:

$$\frac{1}{\nu} = \frac{K_M}{\nu_{\max}[S]} + \frac{1}{\nu_{\max}}, \tag{1}$$

where v is rate of reaction, v_{max} is the maximum rate of reaction measured under saturated substrate condition, and $[S]$ is the bulk concentration of the substrate. K_M was determined by analysis of the slope and intercept for the plot of the reciprocals of the rate of reaction versus substrate concentration. In this receptor, the K_M of GOD in multilayer-enzyme membrane assembled via the layer-by-layer method or not was 0.2 mmol L^{-1} and 2.1 mmol L^{-1} respectively. Therefore, the layer-by-layer assembly of GOD-AuNPs and sol-gel made the K_M small, indicating better affinity of GOD to glucose and higher enzymatic activity to glucose oxidation.

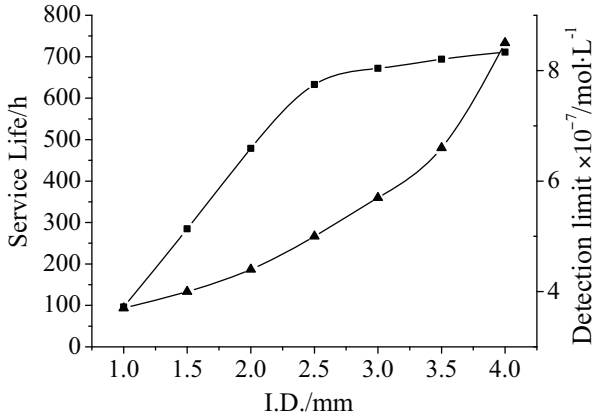

Figure 4. Effect of the receptor i.d. ■ Service life of the receptor ▲ Detection limit of the biosensor.

Optimum of other chemicals

Pump rates were also optimized at the range of 1.2∼3.6 mL min^{-1}. Finally, 2.4 mL min^{-1} was selected as both main pump's and deputy pump's optimal rate at which the relative CL intensity was steady and strong. HCl, H_2SO_4, and NaOH were used respectively as medium to determine the effect of oxidant ($KMnO_4$, $Ce(SO_4)_2$, H_2O_2, and $K_3[Fe(CN)_6]$) to CL. The strongest and steadiest CL intensity was obtained in the $KMnO_4$-HCl system. With the concentrations of HCl at the range of 0.5 to 2.0 mol L^{-1}, the strongest and steadiest CL intensity was obtained at 1.0 mol L^{-1}. The concentration of $KMnO_4$ also had a great effect on CL reaction. The effect of the concentration of $KMnO_4$ on CL intensity was researched in the range of 2.5×10^{-4} to 6.0×10^{-4} mol L^{-1}. Results showed that the optimal concentration of $KMnO_4$ was 5.5×10^{-4} mol L^{-1}. The effect of M2NRASP concentration was investigated in the range 5.0×10^{-6} to 4.0×10^{-5} mol L^{-1}. The results showed that the maximal CL emission peak could be obtained when the concentration of M2NRASP was 2.0×10^{-5}. Therefore, 2×10^{-5} mol L^{-1} M2NRASP was chosen for further study.

Analytical performance

Under the optimal conditions, the calibration graph was linear over the range of 1.0×10^{-6} to 5.0×10^{-2} mol L^{-1}. The linear regression equation was $\Delta I = 714.12 + 139.2c (r = 0.9991)$, where ΔI was the relative

CL intensity and c was the concentration of glucose. The relative standard deviation of this method was below 1.2% in 11 repeated measurements. The limit of detection was 5.0×10^{-7} mol L^{-1}.

Effect of coexisting substances

The effect of various interfering species, which may accompany glucose in human serum, were studied using 1.0×10^{-5} mol L^{-1} glucose. These species were tolerated to a great extent without any special precaution. All other species tested were tolerated at reasonably high concentrations, showing the high selectivity of the proposed method. It is worth mentioning that the influence of the presence of ascorbic acid at its physiologic normal level on the biosensor response to glucose was acceptable. The maximum tolerable concentrations of coexisting substances are shown in Table 1, where the tolerance limit was defined as the concentration of coexisting substances that produced a difference between the signal produced by the coexisting substances solution and the blank signal.

Table 1. Tolerance folds of coexisting substances.

Tolerance folds	Coexisting substances
≥ 1000	K^+, Na, Mg^{2+} , Cl^-, Ca^{2+}, Zn^{2+}, threonine, methionine, valine, isoleucine, lactic acid
≥ 500	Fe^{3+}, tryptophan, lysine, leucine
≥ 100	Histidine, arginine, ascorbic acid

Application to real samples

To investigate the feasibility of the sensing system for analysis glucose in biological samples, glucose concentration in human serum was examined. Six serum samples obtained from hospitalized patients (sample I and sample II from hypoglycemia patients, sample III and sample IV from normal, sample V and sample VI from hyperglycemia patients) were analyzed. All samples were analyzed by the proposed method as well as by a Yellow Springs Instruments (YSI) 2300 glucose analyzer. Results obtained with the proposed method were compared statistically with those obtained with the automated standard colorimetric technique (Table 2).

Conclusions

In this work, M2NRASP, a new type of rhodanine derivative, was synthesized and the $KMnO_4$-HCl-M2NRASP-H_2O_2 CL system was found to be very sensitive to H_2O_2. It is of great importance for widening the area of rhodanine derivatives as CL reagent. A novel glucose-sensitive receptor assembled via the layer-by-layer method was combined with the M2NRASP CL system to establish a biosensor for glucose, and the biosensor was satisfactorily applied to the determination of glucose in human serum. Quantitative detection of glucose ranging from 1.0×10^{-6} to 5.0×10^{-2} mol L^{-1} with a detection limit of 5.0×10^{-7} mol L^{-1} was achieved. The relatively low detection limit, wide detection range, and the simple determination procedure make this CL biosensor method a powerful tool for glucose detection.

Table 2. Determination results of glucose in real samples.

Sample	Glucose concentration (mmol L^{-1})				
	Proposed sensor				Automated standard colorimetric technique
	Added	Found* ± S.D.	R.S.D.%	Recovery%	Found*± S.D.
Sample-1	—	2.776 ± 0.03	1.0	—	2.781 ± 0.3
	10.00	12.625 ± 0.09	0.7	98.4	12.891 ± 0.6
Sample-2	—	2.937 ± 0.04	1.2	—	2.897 ± 0.2
	10.00	13.047 ± 0.1	0.8	101.1	12.913 ± 0.3
Sample-3	—	4.394 ± 0.03	0.7	—	4.433 ± 0.4
	10.00	14.324 ± 0.13	0.9	99.3	14.464 ± 0.3
Sample-4	—	5.891 ± 0.05	0.8	—	5.711 ± 0.5
	10.00	15.851 ± 0.15	0.9	99.6	15.872 ± 0.6
Sample-5	—	6.235 ± 0.05	0.8	—	6.331 ± 0.4
	10.00	16.265 ± 0.15	1.0	100.3	16.472 ± 0.2
Sample-6	—	6.433 ± 0.08	0.8	—	6.3701 ± 0.5
	10.00	16.293 ± 0.14	0.9	98.6	16.4357 ± 0.6

*Average of 11 measurements.

Acknowledgement

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