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Chemiluminescence flow biosensor for glucose based on gold nanoparticle-enhanced activities of glucose oxidase and horseradish peroxidase

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ABSTRACT

In this work, a novel chemiluminescence (CL) flow biosensor for glucose was proposed. Glucose oxidase (GOD), horseradish peroxidase (HRP) and gold nanoparticles were immobilized with sol–gel method on the inside surface of the CL flow cell. The CL detection involved enzymatic oxidation of glucose to D-gluconic acid and H_2O_2, and then the generated H_2O_2 oxidizing luminol to produce CL emission in the presence of HRP. It was found that gold nanoparticles could remarkably enhance the CL respond of the glucose biosensor. The enhanced effect was closely related to the sizes of gold colloids, and the smaller the size of gold colloids had the higher CL respond. The immobilization condition and the CL condition were studied in detail. The CL emission intensity was linear with glucose concentration in the range of 1.0×10^{-5} mol L^{-1} to 1.0×10^{-3} mol L^{-1}, and the detection limit was 5×10^{-6} mol L^{-1} (3σ). The apparent Michaelis–Menten constant of GOD in gold nanoparticles/sol–gel matrix was evaluated to be 0.3 mmol L^{-1}, which was smaller than that of GOD immobilized in sol–gel matrix without gold nanoparticles. The proposed biosensor exhibited short response time, easy operation, low cost and simple assembly, and the proposed biosensor was successfully applied to the determination of glucose in human serum.

1. Introduction

The emergence and recent advance of nanoscience and nanotechnology open new opportunities for the application of nanoparticles in bioanalysis (Zhu et al., 2004; Burda et al., 2005; Gao et al., 2007). This could be attributed to the unique chemical and physical properties of nanoparticles (Cao et al., 2002). In particular, gold nanoparticles attract great scientific and technological interest because of easy preparation, good biocompatibility and relatively large surface-to-volume ratio (Storhoff et al., 1998). The similar dimensions of enzymes and gold nanoparticles enable the interaction of enzymes and gold nanoparticles, and enzymes could easily absorb on the surface of gold nanoparticles. While, in general, the consequent changes in the activity of proteins upon binding to nanoparticles can be seen as a drawback or a potential source of nanoparticle toxicity, there is a potential positive outcome too. Promising uses of nanoparticles include increasing protein stability toward enzyme degradation and increasing the activity of enzymes via immobilization at surfaces (Lynch and Dawson, 2008), which are very useful for constructing enzyme-based biosensors. Gold nanoparticles have been used in many enzyme-based biosensors (Jia et al., 2002; Xiao et al., 1999; Guo and Wang, 2007; Feng et al., 2005; Zhao et al., 2008). However, the most attention has been paid to the construction of the third-generation electrochemical biosensor, which is based on the direct electron transfer between a protein and the electrode (Xiao et al., 1999; Guo and Wang, 2007; Feng et al., 2005; Zhao et al., 2008).

Chemiluminescence (CL) detection is becoming increasingly important in various fields because of the very low detection limit, rapidity and wide linear working range that can be achieved using relatively simple instrumentation. There are some reports in the literature describing the catalytic effect of gold nanoparticles on the CL reaction of luminol in the presence of some oxidants such as hydrogen peroxide, ferricyanide and dissolved oxygen (Zhang et al., 2005a; Duan et al., 2007; Safavi et al., 2008). Moreover, gold nanoparticles are used as label and applied in CL immunoassay (Fan et al., 2005; Li et al., 2005). Nowadays, enzyme-based CL biosensor is known to be a powerful analytical technique because it combines the enzyme specificity with the sensitivity and convenience of CL detection to facilitate analysis (Zhang et al., 2005b; Li et al., 2001, 2002, 2008; Fang et al., 1997). However, to our best knowledge, gold nanoparticles have not been used in the enzyme-based CL biosensor.

Since 1990, encapsulation of enzymes and other biomacromolecules in inorganic sol–gel materials has drawn great interest because of the potential applications in optical and electrochemical

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biosensors (Braun et al., 1990; Jia et al., 2002; Wei et al., 2002; Gupta and Chaudhury, 2007). Sol–gel technology offers a better way to prepare a three-dimensional network suited for the immobilization of enzymes. The encapsulated enzymes retain the same functionality but usually have higher thermal, storage and operational stability in comparison with their counterparts in solution. However, the apparent activity of an entrapped enzyme is often hindered by diffusion of analyte and product in sol–gel matrices (Wei et al., 2002; Shen and Kostic, 1997). Therefore, a suitable method for increasing the apparent activity of enzymes in sol–gel network is required for the development of biosensor.

In this work, we explore the feasibility of using gold nanoparticles to increase the activity of encapsulated enzymes in sol–gel network. Glucose oxidase (GOD), one enzyme of the most widely studied (Raba and Mottola, 1995), is inexpensive and practically applied in clinical and pharmaceutical analysis. So it is chosen as a model enzyme system. GOD and horseradish peroxidase (HRP) firstly interacted with gold nanoparticles, and then the GOD and HRP absorbed on the surface of gold nanoparticles are co-immobilized on the inside surface of the CL flow cell. The CL detection involved the enzymatic oxidation of glucose to D-gluconic acid and H_2O_2, then the generated H_2O_2 oxidizing luminol to produce CL emission in the presence of HRP. The experimental results showed that the response of the biosensor was enhanced more than 20-fold in the presence of 8 nm Au nanoparticles. The proposed biosensor exhibited high sensitivity, easy operation, low cost and simple assembly. On the other hand, the combination of the biosensor with flow-injection analysis made it possible to control all the stages of the reagent additions, measure the enzyme activity, improve the sample throughput and achieve the completely automated determination along with sensitive detection (Ojeda and Rojas, 2006), quick response and repeated use of the immobilized enzyme.

2. Experimental

2.1. Chemical and reagents

Luminol stock solution (2.5×10^{-2} mol L^{-1}) was prepared by dissolving 4.43 g luminol (>95%, Shaanxi Normal University, Xi'an, China) in 20 mL of 0.10 mol L^{-1} NaOH and then diluting to 1 L with water. Glucose oxidase (Type X-S from *Aspergillus niger*, 185 U mg^{-1}) and horseradish peroxidase (Type VI, 330 U mg^{-1}) were obtained from Sigma (St. Louis, MO, USA). β-D-Glucose was purchased from Xi'an Reagent Plant (Xi'an, China). Chloroauric acid ($HAuCl_4$) was purchased from Shanghai Chemical Reagent Company (Shanghai, China). Tetraethyl orthosilicate (TEOS) was obtained from Tianjin Kermel Chemical Reagent Co. Ltd. (Tianjin, China). Sodium citrate was purchased from Beijing Chemical Reagent Company (Beijing, China). All other chemicals were of analytical reagent grade and used without further purification. The water used was deionized and doubly distilled.

2.2. Apparatus and manifold

The flow system employed in this work is shown in Fig. 1. A peristaltic pump was used to deliver all flow streams at a flow rate of 3.0 mL min^{-1} (per tube). PTFE tube (0.8 mm i.d.) was used as connection material in the flow system. 120 μL of mixture solution of sample and luminol was injected by a six-way injection valve into the carrier stream. A U-shaped colorless glass (length: 100 mm, i.d.: 3 mm) was used as the CL flow cell. The GOD and HRP absorbed on the surface of gold nanoparticles are co-immobilized by sol–gel method on the inside surface of the CL flow cell. The CL flow cell was located directly facing the window of the CR-105 photomultiplier tube (Hamamatus, Japan). The CL signal produced in the flow cell was detected and recorded with a computerized ultraweak luminescence analyzer (type BPCL, manufactured at the Institute of Biophysics, Academia Sinica, Beijing, China). The signal was recorded using an IBM-compatible computer, equipped with a data acquisition interface. Data acquisition and treatment were performed with BPCL software running under Windows 95. Absorption spectra were recorded on a TU-1901 UV-Vis Spectrophotometer (Beijing Purkinje General Instrument Co., Ltd., Beijing) at room temperature. Transmission electron microscopy (TEM) measurements were made on a Hitachi H-600 transmission electron microscope operated at an accelerating voltage of 200 kV.

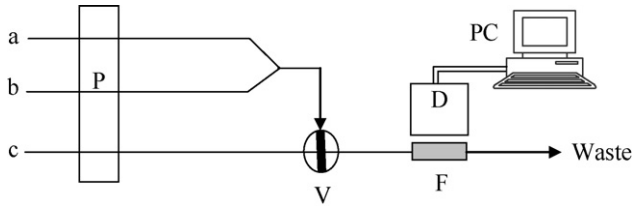

Fig. 1. Schematic diagram of the flow-through biosensor for the determination of glucose: (a) sample; (b) luminol; (c) buffer solution; P: peristaltic pump; V: injection valve; F: flow cell immobilized HRP, GOD and gold nanoparticles; D: detector; PC: personal computer.

2.3. Preparation of gold nanoparticles

All glassware used in the following procedure was cleaned in a bath of freshly prepared 3:1 HNO_3–HCl, rinsed thoroughly in water and dried in air. Gold nanoparticles were prepared according to the published protocol (Grabar et al., 1995). 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, which was stored in the refrigerator (4 °C) and ready for use. The different molar ratios of $HAuCl_4$/sodium citrate were used for the preparation of different nanometer-sized gold nanoparticles. The size of gold nanoparticles was measured by using TEM.

2.4. Preparation of glucose-sensitive membrane

The preparation procedure of the sol–gel stock solution was similar to that proposed by Parang et al. (1994). 2.2 mL TEOS, 0.7 mL H_2O and 50 μL HCl (0.1 mol L^{-1}) were mixed in a glass vial. The mixture was stirred for 3 h and then a clear sol–gel stock solution was obtained. The stock solution was stored in a refrigerator at 4 °C. The HRP solution was prepared by dissolving 10.8 mg HRP in 1 mL phosphate buffer (pH 7.4), and the GOD solution was prepared by dissolving 5.4 mg GOD in 1 mL phosphate buffer (pH 7.4). 30 μL Au colloid solution, 5 μL GOD solution and 5 μL HRP solution were mixed. After about 20 min, the mixed solution of Au colloids and enzyme solutions was added into 0.2 mL sol–gel stock solution. By the spreading method, the mixture was coated on the inside surface of a U-type glass tube (length 100 mm, i.d. 3.0 mm), then was allowed to polymerize and become a gel at room temperature for about 40 min. So, the sol–gel was stuck tenaciously to the inner surface of the U-type glass tube to form a glucose-sensitive membrane. In order to prevent the glucose-sensitive membrane cracking through rapid evaporation, both ends of the U-type tube were sealed during gels aging. Conservation and storage (after gelation) were carried out at 4 °C.

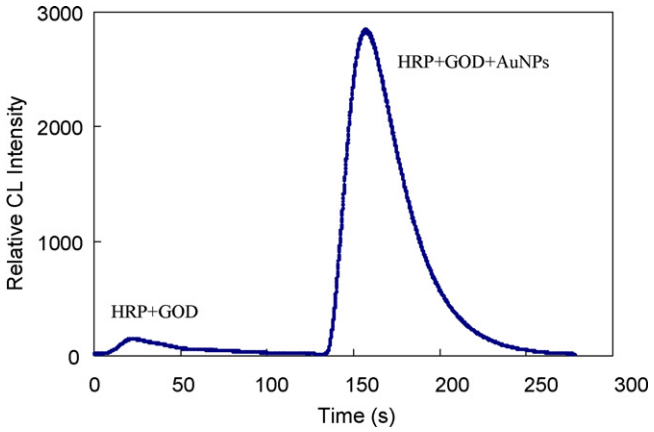

Fig. 2. Observed CL response of this biosensor in the presence and absence of gold nanoparticles (AuNPs).

2.5. Procedures

Flow lines were inserted into the luminol solution, sample solution and phosphate buffer solution, respectively. The pump was started to wash the whole system until a stable baseline was recorded. 120 μL of the mixture solution of sample and luminol was injected into carrier stream (phosphate buffer solution). The stream reached the CL flow cell (enzyme reactor), producing CL emission. When the reaction conditions were optimized, the CL relative intensity corresponded to the normalized maximum light intensity. The concentration of sample was quantified by the peak height of the CL intensity.

3. Results and discussion

3.1. Enhancement effect of gold colloids

The effect of gold colloids on the biosensor was investigated. As shown in Fig. 2, the CL signal was remarkably enhanced by gold colloids. Blank experiments were also carried out. When $HAuCl_4$ and sodium citrate solutions (used for synthesizing gold nanoparticles) were added into sol–gel solution in the presence of GOD and HRP, the enhancement effect on the CL response was not found. In addition, we compared the CL intensities in the presence of different nanometer-sized gold colloids (8 nm, 20 nm and 36 nm). The experimental results showed that the enhanced effect was closely related to the sizes of gold colloids, and for the studied gold colloids with different three sizes, the most intensive CL signal was obtained for gold colloids of 8 nm in diameter. Therefore, the size-related enhancement of gold colloids was ascribed to the gold nanoparticles but not concomitant species such as $HAuCl_4$ and sodium citrate. It was reported that gold nanoparticles could catalyze the CL reaction between luminol and H_2O_2 in solution (Zhang et al., 2005a). In order to test if the immobilized gold nanoparticles in sol–gel could catalyze the CL reaction, 8 nm gold nanoparticles without HRP were immobilized in the CL flow cell with sol–gel method. When luminol and H_2O_2 passed through the CL flow cell, no significant enhancement effect on the CL response was found, which showed no catalytic effect of the immobilized gold nanoparticles in sol–gel matrix on the CL reaction. It was obvious that the enhancement effect on the CL response was also related to the enzymes. As well known, the interaction between protein molecules and Au colloid particles is very strong due to the very high surface-to-volume ratio of gold colloid particles and their high surface energy (Zaitoun, 2005). In the presence of gold nanoparticles, GOD and HRP could adsorb to the gold nanoparticles. What is more, enzymes adsorbed on gold nanoparticles retain their activity because colloidal gold provides an environment similar to the native environment of redox proteins (Crumbliss et al., 1992). This is the main reason why the biosensor's response could be enhanced in the presence of gold particles. On the other hand, the intensity of the CL response depended on the size of gold colloids, and the smaller the size of gold colloids had the higher CL intensity, which is in agreement with that on the gold colloids and HRP-modified gold electrode (Xiao et al., 1999). For gold particles of nanometer scale, it has been widely accepted that they have some important size-dependent properties due to the quantum size effect (Chen et al., 1998). Gold nanoparticles show the electron injection effect and electron withdraw effect (Jiang, 2000). Surface of metallic cluster is always electron deficient, and redox potential of nanometer particles is relative high and is closely related to particle size. In this biosensor, gold nanoparticles, as electron acceptor, could adsorb electrons from the reactive center of reduced GOD (or HRP) molecules and accelerate them return to the oxidized form; at the same time, gold nanoparticles, as electron donor, could transfer electron to oxygen because of its electron withdraw effect. The reactivity of GOD and HRP would then be increased. When amount of atoms contained in one particle decreases, the redox potential of the particle increases (Chen et al., 1998). In this system, the smaller particles (8 nm) were obviously much more able to increase the enzymatic activity. Therefore, 8-nm gold nanoparticles were chosen as the immobilized matrix.

3.2. Characteristics of the UV–vis absorbent spectra

UV–vis spectrum is a useful conformational probe for monitoring the possible conformational changes in the protein secondary structure, which can provide information about possible denaturation of enzyme (Zhao et al., 2008). In order to investigate the interaction between gold nanoparticles and enzymes, we measured the UV–vis absorbent spectra of this system. Fig. 3 shows the UV–vis absorbent spectra recorded from gold nanoparticles (curve a), GOD–HRP (curve b), and the mixture of the gold nanoparticles and GOD–HRP (curve c). The peak at about 280 nm is corresponding to the amino acid residues in proteins, and the peak at about 400 nm is corresponding to the heme group of HRP (Zaitoun, 2005). AuNPs (~8 nm) in aqueous solution are stabilized against aggregation due to the negative capping agents (citrate ions) and have a surface plasma resonance absorption peak at about 520 nm and appear pink (Wei et al., 2007). The enzymic peaks at 280 nm and 400 nm can be clearly seen in the mixture of the gold nanoparticles and GOD–HRP. The absorb peaks of enzymes did not

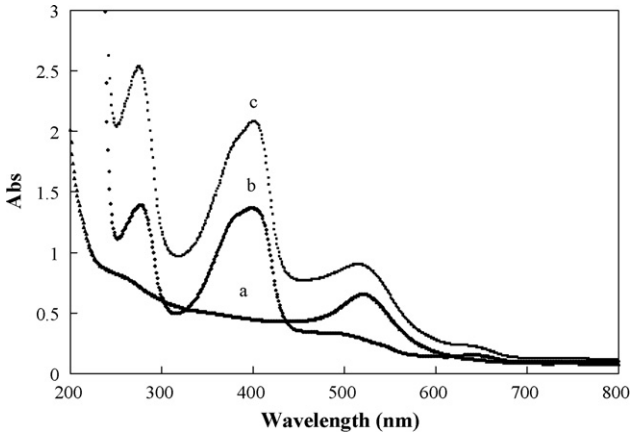

Fig. 3. UV–vis absorbent spectra. (a) Gold nanoparticles, (b) HRP + GOD, (c) HRP + GOD + gold nanoparticles.

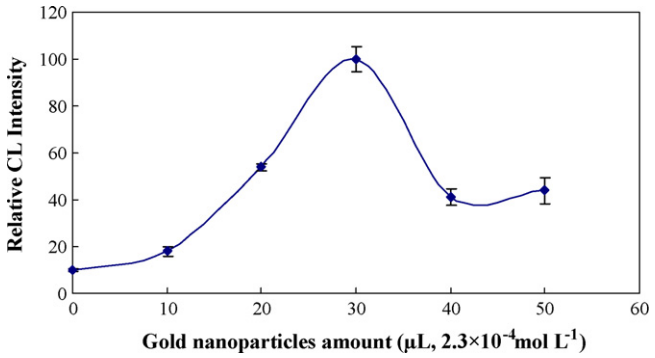

Fig. 4. Effect of amount of gold nanoparticles on the CL intensity. Luminol, 5×10^{-4} mol L^{-1}; pH, 8.0.

shift after interacting with gold nanoparticles. These spectral characteristics indicate that the secondary structure of the GOD and HRP is maintained in the presence of gold nanoparticles, which is useful to keep the native activity of enzymes. So, gold colloids have good biocompatibility for GOD and HRP.

3.3. Optimization of the immobilization condition of enzyme

The used amount of gold nanoparticles is a key factor in this enzyme-based biosensor. The effect of the different amounts of 8 nm gold nanoparticles on the CL intensity was investigated. The experimental results (Fig. 4) showed that the CL intensity firstly increased with increasing amount of gold nanoparticles, probably because the amount of the absorbed enzymes increased. However, above the amount of 30 μL, the CL intensity declined probably because of discontinuous assembly of enzymes on too many gold nanoparticles or mass-transfer processes of substrate and product. Then, the amount of gold nanoparticles of 30 μL (2.3×10^{-4} mol L^{-1}) was chosen as optimum.

In this biosensor, the two enzymes, HRP and GOD, were co-immobilized in the gel membrane. HRP and GOD have very different kinetic characteristics, which have been studied extensively (Mackey et al., 2007). HRP has very fast reaction kinetics and substrate turnover rate. By comparison, GOD is significantly slower. These substrates and products are produced at different rates and are subjected to different diffusion processes. Obtaining the optimum performance of the sensor response will, in one instance at least, depends on the correct ratio of these two enzymes. We investigated the CL respond at different mass ratios of HRP to GOD. The experimental results showed that the higher the ratio of HRP to GOD could result in the higher the CL intensity. At higher ratios of HRP to GOD, the product (hydrogen peroxide), generated in the GOD-catalyzed glucose oxidation, can be consumed more efficiently in the HRP-catalyzed CL reaction. For the biosensor, 2:1 was used as the mass ratio of HRP to GOD. On the other hand, the amount of sol–gel also influenced the sensitivity of the biosensor. Too less sol–gel solution could not encapsulate all the enzymes, and too more sol–gel solution would result in the thicker membrane, which is unfavorable for the diffusion of analyte and product in sol–gel matrices. We used 0.2 mL sol–gel stock solution in this biosensor.

3.4. Optimization of CL detection system

As the luminescence reagent in this system, luminol concentration affects the biosensor response. The effect of luminol concentration in the range of 5×10^{-6} mol L^{-1} to 2×10^{-3} mol L^{-1} was investigated. The result showed that the optimal concentration of luminol was 5×10^{-4} mol L^{-1}, which agreed with our previous study (Li et al., 2008).

In this CL flow system, the carrier solution (of phosphate buffer solution) is the medium of enzyme reaction and also is the medium of the CL reaction. There are two factors that determine the overall response of the biosensor: the influence of pH on the enzyme activity and the effect of pH on the generated CL signal. In view of the nature of luminol CL reaction, which is more favored under basic conditions (pH 10–11), an alkaline medium would improve the sensitivity of the system. However, the optimal pH value for HRP and GOD is 6–7. So, the effect of medium pH on the biosensor was investigated. The CL intensity reached the highest value at pH 8.5, which agreed with our previous results (Li et al., 2001). As such, the reaction was performed at pH 8.5 throughout this study.

Flow rate is a key factor for this flow sensor. The influence of flow rate on the sensor response was investigated. It was found that the lower flow rates with longer contact time could result in an enzymatically sufficient reaction; but on the other hands, lower flow rates were unfavorable for sensitivity because the luminol–H_2O_2 CL reaction was a fast process, and also, a lower flow rate would cause a longer response time. When the flow rate was too higher (>3.0 mL min^{-1}), a smaller amount was produced in the enzymatic reaction because of shorter contact time so that the sensitivity of the sensor was lower. Finally, a flow rate of 3.0 mL min^{-1} was selected as optimum for high CL intensity and rapid response.

3.5. Performance of biosensor for glucose measurements

Under the selected conditions given above, the calibration graph of emission intensity (I, mV) versus glucose concentration was linear in the range of 1.0×10^{-5} mol L^{-1} to 1.0×10^{-3} mol L^{-1}, and the detection limit was 5×10^{-6} mol L^{-1} (3σ). The regression equation was $I = 16.7C \times 10^4 + 63.8$ (where C is the glucose concentration, mol L^{-1}) with a correlation coefficient of 0.9965. A complete analysis, including sampling and washing, could be performed in 80s with a relative standard deviation of less than 2% for 1×10^{-3} mol L^{-1} glucose ($n = 9$). Five standard glucose solutions (1.0×10^{-5} mol L^{-1}, 5.0×10^{-5} mol L^{-1}, 1.0×10^{-4} mol L^{-1}, 5.0×10^{-4} mol L^{-1} and 1.0×10^{-3} mol L^{-1}) were measured continuously (three replicates for one solution) with this flow-through biosensor, and the results are shown in Fig. 5.

The lifetime of the biosensor was studied by recording 100 successive assays of 1×10^{-4} mol L^{-1} glucose, and the relative standard deviation was less than 5%. After 150 successive assays, a 35% decrease of the signal was observed, probably because of leaking

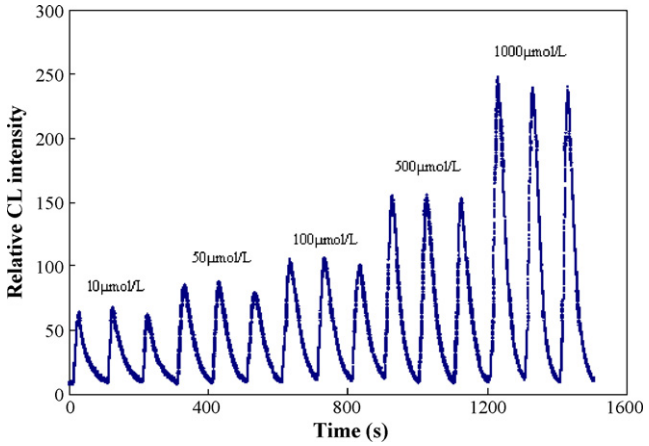

Fig. 5. Results of a series of measurements made with the biosensor.

Table 1
Results of analysis of glucose in serum

Sample	Proposed method (mmol L^{-1})[a]	Spectrophotometric method of hospital (mmol L^{-1})
Serum 1	7.7 (±1.3%)	8.0
Serum 2	4.4 (±1.3%)	4.5
Serum 3	5.3 (±1.1%)	5.5

[a] Average of three replicates (±RSD).

HRP and GOD from the sol–gel membrane. So, the one enzyme reactor could be reused ~100 times. Moreover, the used amount of enzymes in this CL biosensor of glucose is 10th of the enzyme amount in the previous reported CL biosensor of glucose (Li et al., 2001), and the enzyme reactor is inexpensive. On the other hand, it is very easy to prepare and change the enzyme reactor.

The influence of the presence of reducing species at their physiologic normal levels on the biosensor response to glucose (5.6×10^{-3} mol L^{-1}) was also examined. Their interferences were estimated as a percentage of decrease (or increase) in the biosensor signal recorded in the presence of interferents, compared to the response to 5.6×10^{-3} mol L^{-1} glucose alone. The influence of ascorbic acid (5.6×10^{-6} mol L^{-1}), reduced glutathione (5.6×10^{-5} mol L^{-1}), L-cysteine (1.0×10^{-5} mol L^{-1}), and *p*-acetaminophenol (5.6×10^{-5} mol L^{-1}) and uric acid (1.0×10^{-4} mol L^{-1}) to the glucose response were acceptable, namely 3–7%. Ascorbic acid, *p*-acetaminophenol and uric acid gave negative interference possibly because the reducing species can easily reduce hydrogen peroxide produced from the enzymatic reaction. L-cysteine and reduced glutathione gave positive interference possibly because the two thiol-containing compounds could enhance the CL reaction of luminol and H_2O_2 (Du et al., 2001).

3.6. Sample analysis

Serum samples from some volunteers were collected from Shaanxi Normal University Hospital and used as testing samples. The fresh serum samples were first analyzed with a BT3000 Biochemical Analyzer, which used spectrophotometric method combined with the enzymic reaction of GOD and HRP. The samples were then re-assayed with the proposed biosensor. The samples were diluted 100 times by water to yield testing sample solutions. The response of the sample solutions was measured and compared with that of a set of glucose standard solutions. The results are shown in Table 1.

3.7. Determination of the apparent Michaelis–Menten constant

The apparent Michaelis–Menten constant ($K_{M,app}$), which gives an indication of the enzyme–substrate, can be obtained from the Michaelis–Menten equation (Zhang, 2005): $v = v_{max}[S]/(K_{M,app} + [S])$, 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. The Michaelis–Menten equation is transferred to the following form:

$$\frac{1}{v} = \frac{K_{M,app}}{v_{max}[S]} + \frac{1}{v_{max}}$$

the $K_{M,app}$ was determined by analysis of the slop and intercept for the plot of the reciprocals of the rate of reaction versus substrate concentration. In this system, the $K_{M,app}$ of GOD with and without gold nanoparticles in sol–gel matrix was 0.3 mmol L^{-1} and 2.0 mmol L^{-1}, respectively. In the presence of gold nanoparticles, the $K_{M,app}$ became small, indicating better affinity of GOD to glucose and higher enzymatic activity to glucose oxidation in the presence of gold nanoparticles.

4. Conclusions

In this paper, gold nanoparticles firstly were introduced to co-immobilize HRP and GOD in sol–gel matrix for fabricating CL biosensor. The experimental results showed that 8 nm gold particles could remarkably enhance the CL respond of the glucose biosensor. The resulting flow CL biosensor for glucose exhibits good analytical performance: fast response, high sensitivity, easy operation, low cost and simple assembly. Moreover, the system for glucose can be readily adapted to other analytes (e.g. uric acid, lactate) by varying the enzymes.

Acknowledgments

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