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ORIGINAL PAPER

Development of a novel enzyme reactor and application as a chemiluminescence flow-through biosensor

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Abstract A novel enzyme reactor was prepared using calcium alginate fiber (CAF) and amine-modified nanosized mesoporous silica (AMNMS) as a support. Combination of the adsorption of the enzyme on AMNMS with the cage effect of the polymer greatly increases the catalytic activity and the stability of the immobilized enzyme. It was shown that the lifetime, stability, and catalytic activity of the enzyme reactor were greatly improved by incorporating AMNMS into CAF to efficiently encapsulate the enzyme. Glucose oxidase was chosen as a model enzyme to explore the possibility of using CAF–AMNMS as a matrix for enzyme immobilization in the design of a chemiluminescence (CL) flow-through biosensor. The sensitivity of the flow-through biosensor combined with a novel luminol-diperiodatonickelate CL system was higher than for other reported CL biosensors. The proposed biosensor exhibits short response time, easy operation, long lifetime, high catalytic activity, high sensitivity, and simple assembly.

Keywords Chemiluminescence · Flow-through biosensor · Calcium alginate fiber · Amine-modified nanosized mesoporous silica · Glucose oxidase · Glucose

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Introduction

Biosensors have attracted much attention in recent years because of potential applications in the clinical diagnostics, environmental monitoring, pharmaceutical, and food processing industries thanks to their fast response and ease of operation [1, 2]. Free enzyme is not stable and cannot be easily reused. Enzyme immobilization techniques can improve enzyme stability and permit enzyme reuse. For enzyme-based biosensors, the biological recognition molecule is an enzyme, which can catalyze many chemical reactions in living systems. Enzymes may be immobilized by a variety of methods, which can be broadly classified as physical (e.g., adsorption, microencapsulation, membrane reactor confinement), where weak interactions between the support and the enzyme exist, and chemical (e.g., covalent attachment, cross-linking), where covalent bonds are formed with the enzyme [3]. But all the methods have advantages and drawbacks. Adsorption is simple, cheap, and effective but frequently reversible, covalent attachment and cross-linking are effective and durable, but expensive and easily worsen the performance of the enzyme, and in membrane reactor confinement, entrapment, and microencapsulation, diffusional problems are inherent. Therefore, immobilized enzymes often fail to retain their native stability and activity to a certain degree.

Alginate is one of the most promising natural polymers used in enzyme immobilization [4–9] for applications in a variety of fields because enzymes can retain their activity in alginate hydrogels thanks to its swollen and nontoxic environment. The size of the beads is an important factor for applications of calcium alginate, because it has been reported that smaller beads are more biocompatible than larger beads [10] and lower shear forces due to reduced size may increase their long-term stability [11]. In this work, calcium alginate fiber (CAF) about 60 μm in diameter was

prepared to immobilize an enzyme by injecting alginate solution into calcium chloride solution with a pinhead inserted in calcium chloride solution. In previous work it was demonstrated that enzyme leakage is always a great disadvantage when a calcium alginate gel matrix is used in enzyme immobilization [12–14].

The potential applications of nanosized mesoporous silica (MS) in adsorption and catalysis have attracted much attention owing to its high specific surface area, uniform mesoporous channels, and excellent mechanical stability. MS materials provide tunable and uniform pore system, functionalizable surfaces, and restricted nanospaces for enzyme immobilization. Recently, many research groups have realized enzyme immobilization on MS which showed improvement of enzyme stability, catalytic activity, product specificity, and resistance to extreme environmental conditions [15–18]. The surface functional groups play important roles in enzyme immobilization by changing the surface charge of MS for controlling electrostatic interactions with the adsorbed enzyme. For example, amine groups on the MS surface will bind to negatively charged enzymes, whereas carboxylate groups will encapsulate positively charged enzymes [19]. Therefore, a novel enzyme reaction incorporating amine-modified nanosized MS (AMNMS) into CAF was prepared to efficiently encapsulate an enzyme, which could combine the adsorption of the enzyme on AMNMS with the cage effect of the polymer to potentially increase the catalytic activity and stability of the immobilized enzyme.

The chemiluminescence (CL) method is now used for a wide range of quantitative applications and offers the advantages of low detection limits, wide linear dynamic range, and rapid response with simple instrumentation. Many enzyme-based CL biosensors have been reported [20–25]. In this work, we explored the feasibility of using AMNMS to increase the activity and stability of enzyme encapsulated in CAF. Glucose oxidase (GOD; pI 4.0) was chosen as a model enzyme to prepare the enzyme reactor to explore the possibility of using CAF–AMNMS as a matrix for enzyme immobilization in the design of a CL biosensor. The signal of the luminol-diperiodatonickelate (DPN) system which we have reported [26] can be greatly enhanced by H_2O_2. In this work, glucose was determined by the enzymatic oxidation of glucose to d-gluconic acid and H_2O_2; H_2O_2 then reacts immediately with luminol to a generate CL signal in alkaline medium. Hence, a new CL flow-through biosensor with immobilized enzyme was proposed. The current novel CL flow-through biosensor with CAF–AMNMS as the enzyme immobilization platform offers several advantages: (1) enzymes retain their activity in CAF–AMNMS owing to its swollen and nontoxic environment; (2) smaller-diameter CAF offers better transportation [27], dispersion, and mechanical strength, easier implantation, good stability, and potential access to new implantation sites [28]; (3) adsorption of the enzyme on AMNMS combined with the cage effect of the polymer will increase the capability of enzyme immobilization; (4) the huge surface area, biocompatibility, low cytotoxicity, rigid structure, and pore surface of AMNMS improve enzyme stability and catalytic activity [19]; (5) the sensitivity of many glucose sensors reported in recent years was mostly limited to micromolar and rarely reached nanomolar [29–31], but the biosensor possesses excellent sensitivity to determine glucose combined with the novel luminol-DPN system. The combination of the biosensor with flow-injection analysis makes it possible to control all the stages of reagent addition, improve the sample throughput, and achieve completely automated determination along with sensitive detection, quick response, and repeated use of the immobilized enzyme [32]. Therefore, a novel CL flow-through biosensor with high sensitivity, long lifetime, and high catalytic activity for glucose was developed and successfully applied to the determination of glucose in human serum.

Experimental

Chemical and reagents

Luminol stock solution (1.0×10^{-2}M) was prepared by dissolving 1.772 g luminol (purity better than 95%, Shaanxi Normal University, China) in 1 L 0.1 M carbonate buffer; it was left to stand for approximately 24 h before use and was stored in the dark. GOD was obtained from Sigma (St. Louis, MO, USA). d-Glucose, tetraethylorthosilicate (TEOS), cetyltrimethylammonium bromide (CTAB), and anhydrous calcium chloride were obtained from Sinopharm Chemical Reagent Comapny (Shanghai, China). Potassium persulfate was from Shanghai Aijian Chemical Reagent Company, and sodium alginate, potassium hydroxide, hydrogen peroxide, sodium nitrate, sodium periodate, and silver nitrate were from Shanghai Chemical Reagent Company. All reagents were of analytical grade, and deionized and double-distilled water was used throughout.

Apparatus

The flow system employed in this work is shown in Fig. 1. Two peristaltic pumps (Remex Analytical Instrument Company, Xi'an, China) were used to deliver all flow streams. Polytetrafluoroethylene flow tubes (0.8-mm inner diameter) were used to connect all components in the flow system. Injection was done using an eight-way injection valve equipped with a sample loop (90 μL). CAF-or CAF–AMNMS-immobilized GOD was packed into a glass tube (length 55 mm, inner diameter 3 mm). The flow cell was

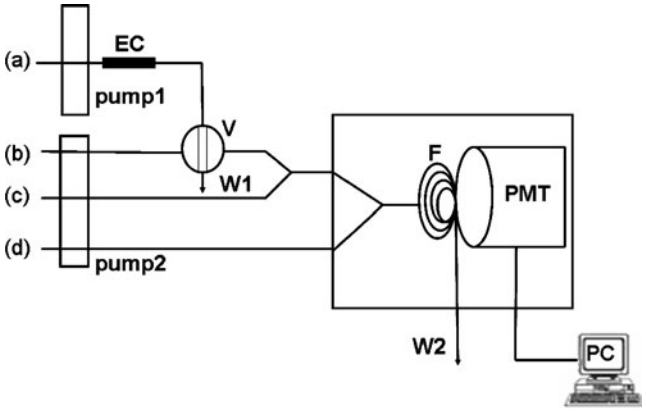

Fig. 1 The flow-through biosensor for the determination of glucose: **a** glucose standard solution or samples, **b** distilled water, **c** luminol solution, **d** diperiodatonickelate (DPN) solution, *EC* enzyme immobilization column, *V* injection valve, *F* spiral glass cell, *PMT* photomultiplier tube, *pump1*, *pump2* peristaltic pumps, *W1*, *W2* waste

made by coiling 20 cm of a colorless glass tube (2-mm inner diameter) into a spiral disk shape and was located directly facing the window of the photomultiplier tube. The CL signal was monitored using an IFFM-A multifunction CL analyzer (Remex Analytical Instrument Company, Xi'an, China). The UV absorbance was detected with a U-3900 spectrophotometer (Hitachi, Japan). Environmental scanning electron microscopy was performed with a Quanta 200 (FEI Japan) scanning electron microscope and transmission electron microscopy was performed with a JEM-2100 transmission electron microscope (JOEC, Japan).

Preparation of nanosized MS

A typical synthetic gel was prepared by adding 5.4 mL TEOS to an aqueous solution containing 1.1 g CTAB, 0.31 g NaOH, and 270 mL deionized water. After the mixture had been stirred for about 2 h at room temperature, the resulting product was filtered, washed three times with distilled water, and dried at ambient temperature, followed by calcination in air at 550 °C for 5 h to obtain nanosized MS [33].

Pore size expansion

Nanosized MS (0.56 g) was added to 2 mL solution mixture containing distilled water, NaCl, LiCl, and KNO_3 in a proportion of 70:20:5:5 (weight), and the resulting mixture was then heated at 300 °C for 2 h to obtain remodeled MS [34]. The remodeled MS was washed with distilled water and dried at ambient temperature.

Preparation of AMNMS

AMNMS was obtained when 0.5 g nanosized MS or remodeled nanosized MS was refluxed in 30 mL dry toluene solution containing 0.5 mL 3-aminopropyltrimethoxysilane (97%, Aldrich) at 100 °C for 12 h [35]. The AMNMS obtained was washed with ethanol and distilled water and dried at ambient temperature.

Enzyme immobilization in AMNMS

A 1-mL volume of 4.5 mg mL^{-1} GOD stock solution [dissolved in 5 mM pH 6.5 tris(hydroxymethyl)aminomethane–HCl buffer] was added to 20 mg AMNMS or remodeled AMNMS in a 5-mL graduated centrifuge tube. The mixture was stirred for 24 h at 4 °C. The AMNMS-or remodeled-AMNMS-immobilized GOD was separated from solution by centrifugation and washed with distilled water.

Immobilization of GOD in CAF

GOD solution (0.5 mL) was mixed with 5 mL 5.0% aqueous sodium alginate solution and stirred for 5 min. The mixed solution was placed in a syringe and injected into 4% calcium chloride solution with a needle (0.45-mm inner diameter) below the surface of the calcium chloride solution, forming CAF with GOD entrapped. The fibers were allowed to harden for 2 h at 4 °C. The fibers obtained were washed and stretched in distilled water, then dried. The dry fibers were cut into 5-cm-long pieces, and were then packed into a glass column filled with a small amount of glass wool at both ends to prevent the fibers from being washed away by the solution. The enzyme reactor was stored at 4 °C until use. The reactor was denoted as sensor A.

Immobilization of GOD in CAF–AMNMS

The AMNMS-or remodeled-AMNMS immobilized GOD was well dispersed in 0.5 mL distilled water by ultrasonic treatment prior to use. Then the mixture solution was mixed with 5 mL 5.0% aqueous sodium alginate solution and the rest of the procedure was as same as that described in "Immobilization of GOD in CAF." The reactor with AMNMS or remodeled AMNMS was denoted as sensor B or sensor C, respectively.

Procedures

During the enzymatic reaction, a glucose molecule was enzymatically oxidized by an oxygen molecule, releasing the stoichiometric equivalent of H_2O_2. The determination of H_2O_2 was based on the CL reaction of luminol–DPN in alkaline medium. The CL flow-through system used for the determination of glucose is shown in Fig. 1. Flow lines were inserted into the luminol solution, DPN solution, carrier stream, and sample solution, respectively. The whole system was then flushed until a stable baseline was

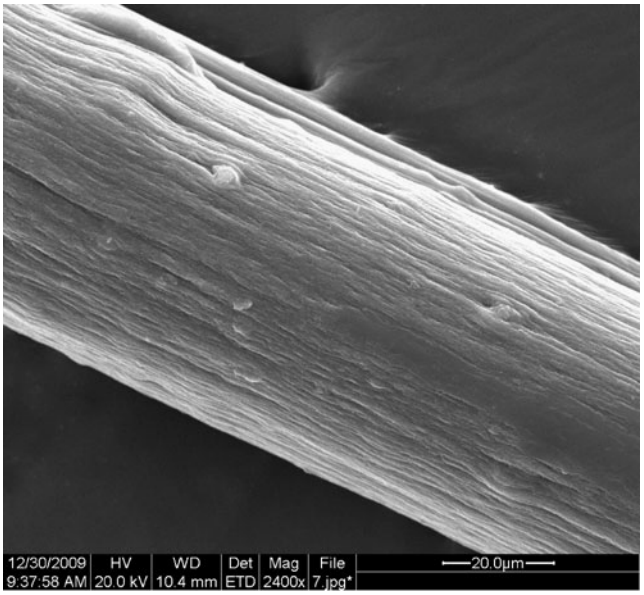

Fig. 2 Scanning electron microscope image of calcium alginate fiber

recorded. When the analyte solution passed through the immobilized GOD column, glucose was oxidized to d-gluconic acid and H_2O_2. Then 90 μL of the H_2O_2 produced was injected into the carrier stream to react with luminol and DPN to produce CL. The CL signal was detected by the IFFS-A multifunction CL analyzer. The concentration of the sample was quantified by the peak height of the CL intensity.

Results and discussion

Characterization of CAF

Alginate beads have been extensively used to entrap enzymes owing to their excellent biocompatibility, non-toxicity, and potential bioactivity. In this work, CAF was used to immobilize the enzyme. Immobilization of enzymes or microorganisms in CAF has two major advantages over immobilization in alginate beads: diffusion in alginate fiber with a smaller diameter is better than that in alginate beads [36] and alginate fibers retain more enzymes compared with alginate beads [37]. The morphology of CAF was examined using environmental scanning electron microscopy (Fig. 2). It was concluded that the diameter of the fiber was about 60 μm. Though enzymes retain their activity in alginate hydrogels thanks to the swollen and nontoxic environment, leakage is a big problem owing to the fact that even large enzymes with a molecular mass of 300 kDa can leak out from calcium alginate [38–40]. In this work, the leakage of GOD was remarkably reduced by immobilization of GOD in CAF–AMNMS compared with immobilization in CAF.

Characterization of AMNMS

Images of AMNMS and remodeled AMNMS were taken using a JEM-2100 transmission electron microscope (Fig. 3). It can be clearly observed that remodeled AMNMS has bigger mesoporous cavities, which were more suitable for enzyme immobilization [34]. The adsorption ability was confirmed by the UV–vis absorption spectra (Fig. 4).

Two biosensors were prepared using AMNMS and remodeled AMNMS combined with CAF to immobilize GOD and were studied for their CL response.

The amount of GOD immobilized in AMNMS

The amount of GOD immobilized in AMNMS or remodeled AMNMS was measured by adding 20 mg AMNMS to 1 mL 4.5 mg mL^{-1} GOD solution and the mixture was stirred for 24 h at 4 °C. The supernatant was separated from the solid material by centrifugation and the amount of immobilized enzyme was calculated from the difference in the absorbance of the supernatant at 450 nm before and after addition of the support (Fig. 4). Unexpectedly, remodeled AMNMS shows a greater adsorption capacity of 60 mg g^{-1} (GOD/AMNMS) than AMNMS (40 mg g^{-1}). It is concluded that remodeled AMNMS has more pores for enzyme immobilization when a mixture of three salts was used to enlarge the pore size of AMNMS. Meanwhile, AMNMS and remodeled AMNMS provide a biocompati-

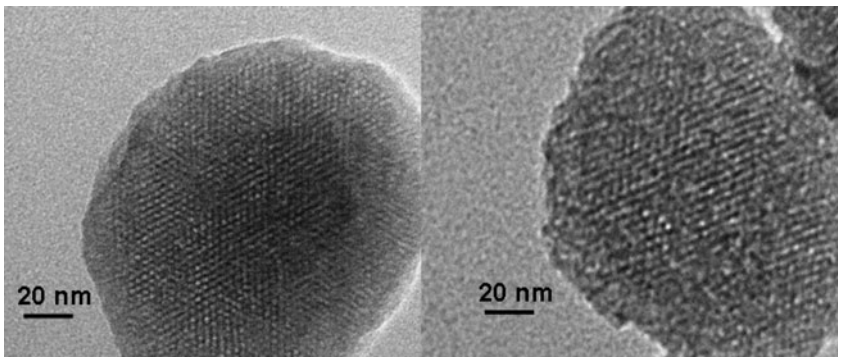

Fig. 3 Transmission electron microscope images of amine-modified nanosized mesoporous silica (AMNMS) (*left*) and remodeled AMNMS (*right*)

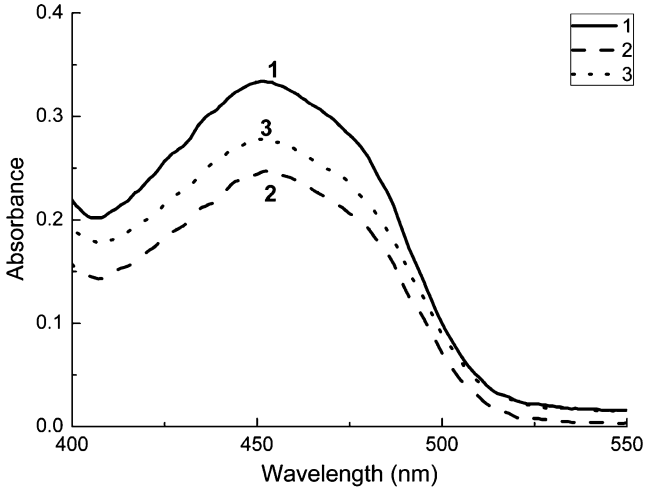

Fig. 4 UV–vis spectra of glucose oxidase (GOD) solution (pH 6.5): *1* 4.5 mg mL^{-1} GOD solution; *2* after adsorption to remodeled AMNMS; *3* after adsorption to AMNMS

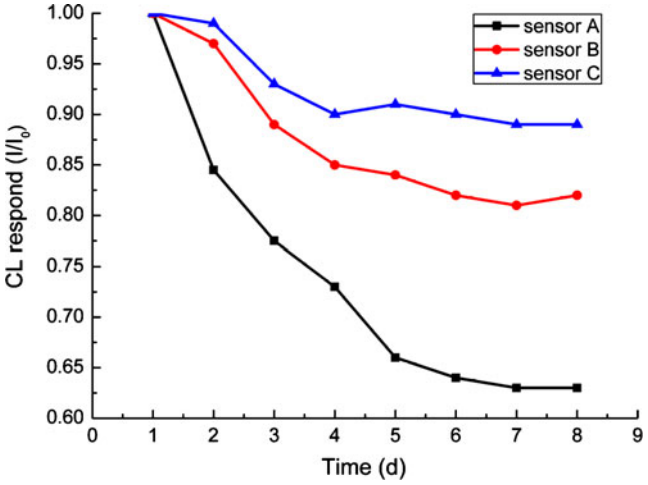

Fig. 5 Variation of the chemiluminescence (*CL*) response with time

bile environment for GOD to keep its bioactivity by virtue of their biochemical inertness and relative mechanical strength [41].

Optimization of the CL detection system

The flow rate of pump 1 is an import factor in the enzymatic oxidation of glucose because the time glucose was allowed to stay in the column controlled the rate of conversion of glucose to the H_2O_2 to be detected. It was found that although a higher CL signal could be obtained at a slower flow rate, the analysis rate might be limited in this condition. To obtain both high sensitivity and a rapid analysis rate, 0.5 mL min^{-1} was finally selected as the optimum flow rate.

The flow rate of pump 2 was also an important factor which influences analytical sensitivity. The effect of the flow rate on CL intensity was examined in the range 0.5–3.5 mL min^{-1}. The results showed that the CL signal increased as the flow rate increased, because the CL reaction is rapid. So a flow rate of 3.5 mL min^{-1}, the maximal flow rate under the present conditions, was selected as optimum.

Lunimol was the luminescent reagent in this system, and its concentration affected the biosensor response. The effect of luminol concentration on the signal-to-noise ratio was investigated for the range 6.0×10^{-8}–1.0×10^{-6}M. It was found that the higher the concentration of luminol is, the greater the light emission that can be obtained, but greater baseline noise was observed with increasing luminol concentration, which leads to lowering of the signal-to-noise ratio. Therefore, 2.0×10^{-7}M luminol was used as an optimum concentration.

The effect of DPN concentration on the CL intensity was examined in the range from 1.0×10^{-5} to 8.0×10^{-4}M. It was found that the CL intensity increased with DPN concentration up to 8.0×10^{-5}M; it decreased thereafter because the higher concentration of DPN resulted in self-absorption. Therefore, 8.0×10^{-5}M DPN was selected.

DPN was used as an oxidant in aqueous alkaline medium. The effect of potassium hydroxide in DPN solution on the CL intensity was examined. The concentration of potassium hydroxide was studied at different concentrations from 5.0×10^{-3} to 4.0×10^{-1}M. It was found that the CL intensity increased with potassium hydroxide concentration up to 8.0×10^{-2}M, and decreased thereafter. To obtain the highest sensitivity and accuracy, 8.0×10^{-2}M potassium hydroxide was selected as the optimum concentration.

Lifetime of the biosensor

The lifetime of the biosensor depended mainly on the enzymatic stability and activity. The storage stability of the

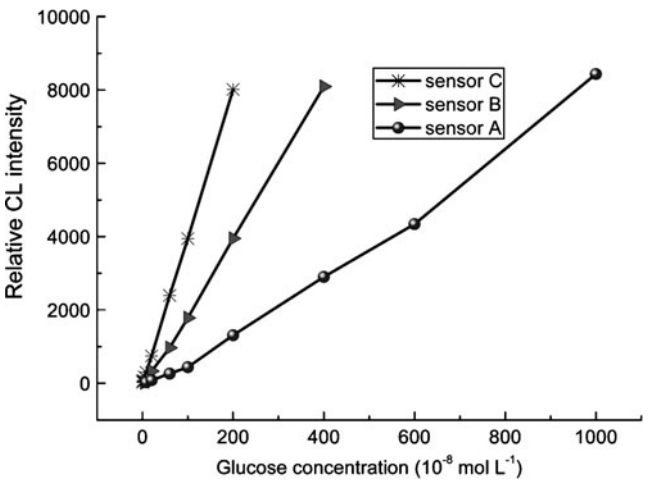

Fig. 6 The CL response of the three sensors; 2.0×10^{-8}M luminol, 8.0×10^{-8}M DPN, 8.0×10^{-2}M KOH

Table 1 The analytical characteristic of the sensors

Sensor	Linear range (M)	Regression equation(C: 10^{-8}M)	Detection limit (3σ, M)
Sensor A	8.0×10^{-8}–1.0×10^{-5}	ΔI=8.3697C-273.48 (R^2=0.993)	2.0×10^{-8}
Sensor B	4.0×10^{-8}–4.0×10^{-6}	ΔI=20.488C-146.62 (R^2=0.9992)	8.3×10^{-9}
Sensor C	8.0×10^{-9}–2.0×10^{-6}	ΔI=39.995C-9.125 (R^2=0.999)	2.2×10^{-9}

sensors at 4 °C was examined by periodically checking their CL response. It was found that during the first 6 days the CL intensity of sensor A, sensor B, and sensor C dropped to approximately 64, 82, and 90% (Fig. 5) of its initial value, respectively. Therefore, incorporating AMNMS into CAF would be an effective approach to efficiently encapsulate enzyme, which could combine the adsorption of the enzyme on AMNMS with the cage effect of the polymer and potentially increase the activity and stability of the immobilized enzyme. The discrimination of sensor B and sensor C is because remodeled AMNMS has larger pores than AMNMS. GOD can be entrapped in the pores of remodeled AMNMS, and cannot easily leak from the support, instead of being immobilized on the surface of AMNMS [34]. After 4 days the response of sensor C for the same glucose solution remained nearly constant over a 3-month storage period. It is suggested that sensor C possesses good stability when the newly immobilized enzyme reactor is stored for 4 days prior to use. But the response of sensor A dropped to approximately 36% after the 3-month storage period. On the other hand, the operational stability of the biosensor was studied by recording more than 100 successive assays of 1.0×10^{-5}M glucose, and the relative standard deviation was 9.5 and 1.4%, respectively, for sensor A and sensor C. So sensor C has a longer lifetime and higher stability.

Interference studies

The effects of foreign substances in serum were tested by analyzing a standard solution of glucose (8.0×10^{-8}M) to which increasing amounts of interfering substances were added. The tolerable concentration ratios with respect to 8.0×10^{-8}M glucose at an interference level of 5% were over 1,000 for Na^+, Ca^{2+}, NO_3^-, NH_4^+, and SO_4^{2-}, 500 for urea, lysine, creatinine, glutamic acid, leucine, serine, asparagine, and oxalate, and 50 for uric acid, lactose, lactic acid, ascorbic acid, and Fe^{3+}. The interference of protein in human serum could be ignored when human serum was ultrafiltered, thus implying that the present method may be directly applied to the determination of glucose in human serum.

Table 2 Figures of merit of comparable chemiluminescence (*CL*) biosensors for the determination of glucose

CL biosensor	Linear range (M)	Detection limit (M)	Reference
Luminol–HRP	1×10^{-6}–1×10^{-4}	5×10^{-7}	[42]
Luminol–HRP–GNPS	1×10^{-5}–1×10^{-3}	5×10^{-6}	[43]
Luminol–$KFe_3(CN)_6$	1.1×10^{-3}–1.1×10^{-1}	1×10^{-4}	[44]
Luminol–HRP–GNPS	1×10^{-5}–6×10^{-3}	5×10^{-6}	[45]
Luminol–HRP	0–1.2×10^{-2}	5×10^{-5}	[46]
Sensor C	8.0×10^{-9}–2.0×10^{-6}	2.2×10^{-9}	Present method

HRP horseradish peroxidase, *GNPS* gold nanoparticles

Performance of the biosensor for glucose measurements

Under the selected conditions given already, the CL responses of sensor A, sensor B, and sensor C (sensor A and sensor C with equal amounts of GOD, sensor B and sensor C with equal amounts of AMNMS) were compared using the curves shown in Fig. 6. Calibration graphs of the relative CL intensity (ΔI) versus glucose concentration were obtained (Table 1) for each sensor described. It is indicated that the responses of sensor B and sensor C, in which AMNMS is employed, are higher than the response of sensor A. The higher responses of sensor B and sensor C are attributed to the huge surface area provided by AMNMS to improve enzyme stability and catalytic activity. AMNMS can adsorb the GOD intensively and prevent enzyme leakage because the MS surface modified with amine groups can bind negatively charged enzyme by electrostatic interaction. The CL response of sensor C is higher than that of sensor B because AMNMS remodeled by increasing the pore size can adsorb more GOD. So sensor C has the highest sensitivity. Several CL biosensors have been reported for glucose analysis, and the figures of merit are shown in Table 2. It is shown that the proposed CL biosensor exhibits the highest sensitivity.

Table 3 Analysis results of glucose in serum

Sample	Proposed method (mM)[a]	*o*-Toluidine method (mM)
Serum 1	4.7 (±4.3%)	4.8
Serum 2	6.8 (±0.6%)	7.0
Serum 3	6.6 (±2.3%)	6.7
Serum 4	3.9 (±4.3%)	4.1
Serum 5	3.9 (±2.5%)	4.0

[a] Average of three replicates (± relative standard deviation)

Sample analysis

The proposed method was utilized for the determination of glucose in human serum. The samples were first analyzed with the *o*-toluidine method (630 nm) [47]. The samples were then reassayed with the proposed biosensor. The samples were diluted with distilled water to yield test sample solutions. The response of the sample solution was measured and compared with that of a set of glucose standard solutions. The results matched well with those obtained with the *o*-toluidine method and are shown in Table 3.

Conclusion

CAF–AMNMS was firstly introduced to immobilize GOD for preparation of a CL flow-through biosensor. Combination AMNMS with CAF efficiently reduced leakage and improved the activity and stability of the immobilized enzyme. The biosensor shows excellent stability with a long shelf life and high sensitivity for glucose. The proposed biosensor possesses higher sensitivity combined with the novel luminol–DPN CL system compared with other CL biosensors. Moreover, the system can be readily adapted to other analytes (such as uric acid and lactate) by varying the enzyme.

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