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Highly sensitive glucose biosensor based on the effective immobilization of glucose oxidase/carbon-nanotube and gold nanoparticle in nafion film and peroxyoxalate chemiluminescence reaction of a new fluorophore

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ABSTRACT

A novel glucose biosensor based on the chemiluminescence (CL) detection of enzymatically generated H_2O_2 was constructed by the effective immobilization of glucose oxidase (GOD)/carbon-nanotubes (CNTs)/gold nanoparticles (GNPs) in nafion film on graphite support. The influences of various experimental parameters such as solution pH, the action time of the enzyme, interferents and the concentration of CL reagents were investigated. Carbon nanotubes and gold nanoparticles offer excellent catalytic activity toward hydrogen peroxide generation in enzymatic reaction between glucose oxidase and glucose, which would enable sensitive determination of glucose.

Under the optimum condition, the linear response range of glucose was found to be 2.25×10^{-6} to 1.75×10^{-4} mol L^{-1}, and the detection limit (defined as the concentration that could be detected at the signal-to-noise ratio of 3) was 1.00×10^{-6} mol L^{-1}. The CL biosensor exhibited good storage stability, i.e., 80% of its initial response was retained after 10 days storage at pH 7.0. The present CL biosensor has been used to determine the glucose concentrations in real serum and urine samples with satisfactory results.

1. Introduction

Quantitative determination of glucose is very important in biochemistry, clinical chemistry, food processing and fermentation [1–8]. To date, the most common glucose determination methods are based on the electrochemical or spectrophotometrical detection of hydrogen peroxide liberated in enzymatic reaction between glucose oxidase (GOD) and glucose [9–17]. However, both methods are subject to several interferences. These interferences include reducing substances such as bilirubin, ascorbic acid, uric acid, and drug metabolites [10,18,19]. Other disadvantage of these methods is lack of sensitivity. These problems seriously limit their application for glucose determination in physiological samples such as urine containing low content of glucose and high amounts of uric acid.

Hence, it is relevant to explore and develop a simple and sensitive method for glucose determination especially in clinical analysis in the presence of species such as ascorbic acid and uric acid.

Peroxyoxalate chemiluminescence (PO-CL) is a powerful technique for the sensitive and some times selective determination of a large variety of analytes, depending on the roles they play in the CL reaction as Fluorophore (Flu) [20–23], catalyst [24], quencher [25–27] or oxidant [28,29]. In general, PO-CL involves the hydrogen peroxide oxidation of an aryl oxalate ester in the presence of a fluorophore (activator). The reaction has been suggested to follow a chemically initiated electron exchange luminescence (CIEEL) mechanism via the formation of a high energy intermediate(s) such as 1,2-dioxetanedione [30,31]. These metastable intermediates form complexes with the Flu so that one electron can be donated to the intermediate [32,33]. This electron is then transferred back to the Flu raising it to an excited state and liberating "light". These steps are depicted in Scheme 1. The light emitted is proportional to the concentration of hydrogen peroxide.

The coupling of PO-CL detection with enzymatic reactions can significantly increase both the sensitivity and selectivity of the glucose determination methods, because CL emission is measured

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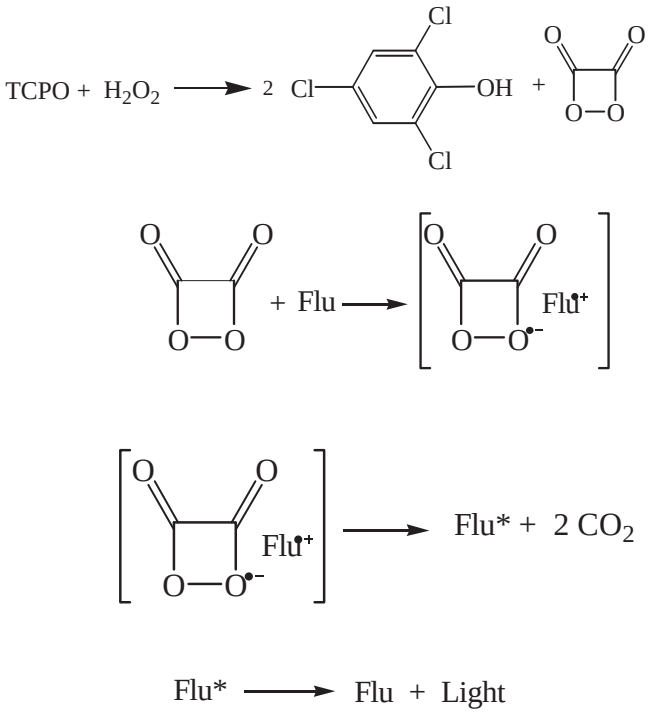


Scheme 1. Common mechanistic pathway for PO-CL reactions.

against a dark background and uric acid does not interfere in glucose determination using PO-CL detection system [34–37].

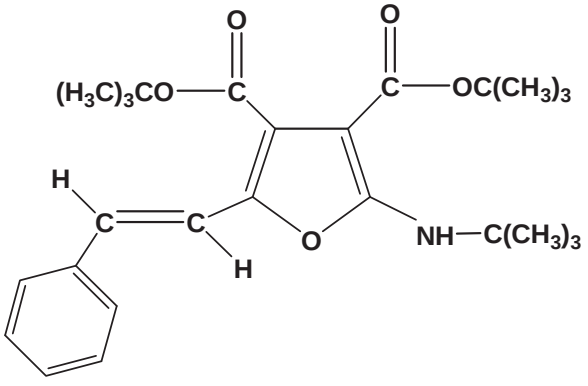
However the overall quantum yield (Φ_{CL}) of PO-CL reactions is directly dependent on the fluorescence quantum yield of the Flu. This dependence could be described by the following equation [38]:

$$\Phi_{CL} = \Phi_{CE} \times \Phi_F \times \Phi_R \quad (1)$$

where Φ_{CE}, Φ_F, and Φ_R represent chemiexcitation quantum yield (the probability of generating an electronic excited state in a reaction), fluorescence quantum yield (the probability of the excited state emitting a photon by a fluorescence rather than decaying by other processes) and reaction quantum yield (the fraction of starting molecules which undergo the luminescent reaction rather than a side reaction), respectively. Thus it is pertinent to design and synthesis new and more fluorescent Fluorophore for sensitive determination of glucose Scheme 2.

In recent years we have studied the PO-CL reaction of several Fluorophores in the presence of the suitable basic catalysts such as sodium salicylate or imidazole [39–41].

In this work we propose a novel highly fluorescent Flu di(tert-butyl)-2-(tert-butylamino)-5-[(E)-2-phenyl-1-ethenyl]



Scheme 2. Structure of Flu.

-3,4-furandicarboxylate for PO-CL detection of H_2O_2 generated in enzymatically oxidation of glucose.

On the other hand, glucose oxidase is an expensive and environment-sensitive material. Thus the enzyme immobilization is considered to be one of the most important issues in designing enzyme-based glucose determination methods. Nafion (perfluorinated sulphonate ionomer), due to its easy fabrication, high resistance to chemical attack, good biocompatibility and high permselectivity toward anionic interferents (nafion film has negative charge due to its sulfonic functional group) is a good choice for immobilization of GOD and construction of glucose biosensors [42–45].

In addition many works demonstrated electrocatalytic effect of carbon nanotubes (CNTs) and gold nanoparticles (GNPs) on enzymatic activity and stability of GOD [46–50] and several mechanisms about the effect of GNPs and CNTs on the GOD activity in terms of a, large surface area, fast mass transport, good electron accepting and good conducting were reported [47–52].

To the best of our knowledge, the PO-CL investigation on GOD activity entrapped in (CNTs)/(GNPs)/nafion film has not been reported. Therefore in this paper a novel glucose biosensor based on the chemiluminescence (CL) detection of enzymatically generated H_2O_2 was constructed by the effective immobilization of GOD/CNTs/GNPs in nafion film on graphite support. Influence of various experimental parameters on glucose sensing, including reaction time, solution pH, interferents and the concentration of CL reagents was investigated. Catalytic activity of CNTs and GNPs on hydrogen peroxide liberated in enzymatic reaction between glucose oxidase and glucose was explored. The constructed CL biosensor was used to determine the glucose concentration in real serum and urine samples with the satisfactory results.

2. Experimental

2.1. Reagents and solutions

Bis(2,4,6-trichlorophenyl)oxalate (TCPO) was prepared from the reaction of 2,4,6-trichlorophenol with oxalylchloride in the presence of triethylamine as described elsewhere [53]. Hydrogen peroxide (Merck; Perhydrol Suprapur, 30% in water) was assayed by permanganate potassium titration [54] and diluted in acetonitrile. Fluorophore di (tert-butyl)-2-(tert-butylamino)-5-[(E)-2-phenyl-1-ethenyl]-3,4-furandicarboxylate was synthesized in our laboratory [55]. The stock solution of Flu (0.005 mol L^{-1}) was prepared in a calibrated 50-mL flask by dissolving an appropriate amount of Flu in acetonitrile and protected from light. Stock solutions of sodium salicylate (0.005 mol L^{-1} in acetonitrile) and the TCPO (0.005 mol L^{-1} in acetonitrile) were prepared shortly before use.

Spherical GNPs were prepared according to the literature [56] by adding sodium citrate solution to a boiling $HAuCl_4$ aqueous solution. The solution was stored in brown glass bottles at 4 °C. The average nanoparticle diameter is 20 nm as measured by transmission electron microscopy (not shown here). MWCNT (multi-walled carbon nanotube was purchased from Aldrich. Hydrogen tetrachloroaurate (III) hydrate ($HAuCl_4 \cdot 3H_2O$), nafion (10 wt%) solution in a mixture of lower aliphatic alcohols and water were obtained from Aldrich. Glucose and Glucose oxidase from Aspergillus niger were purchased from Sigma. Phosphate buffer solutions (0.1 mol L^{-1}) with various pH values were prepared by dissolving an appropriate amount of K_2HPO_4 in water and adjusting the pH values with 0.1 mol L^{-1} HCl or NaOH solutions. All other chemicals were of analytical grade.

2.2. Preparation of nafion/GOD–CNT-GNPs/biosensor

Graphite supports (5 mm × 5 mm × 30 mm) after rinsing thoroughly with doubly distilled water, was sonicated in absolute ethanol and doubly distilled water for about 1 min respectively. Graphite was selected as support because its porous structure strongly holds the nafion film. GOD solution was obtained by dissolving 4.0 mg of GOD in 2 ml of 0.1 mol L^{-1} pH 7 PBS, and the GNPs were used as prepared. 0.2 mg MWCNT was dispersed in 5 ml 5% nafion ethanol solution. A 100 μL nafion ethanol solution (5%) was dropped onto the surface of a cleaned graphite support with a microsyringe and allowed to dry at ambient temperature. Then a 120 μL mixture of GOD, GNPs and CNTs (v/v = 1:1:1) was dropped onto the previous surface and allowed to dry. Finally a 100 μL nafion ethanol solution (5%) was dropped onto the prepared surface to hold the GOD–GNPs–CNTs on the support surface stably and enhance selectivity toward anionic species. The final product is taken as the nafion/GOD–GNPs/CNTs/graphite biosensor. The similar procedures were employed to fabricate the nafion/GOD/graphite, nafion/GOD/GNPs/graphite and nafion/GOD/CNTs/graphite biosensors. All resulting sensors were stored at 4 °C when not in use.

2.3. Chemiluminescence measurements

Chemiluminescence measurements were carried out by a Sirius-tube luminometer (Berthold detection system, Germany) with a photomultiplier tube detector. The temperature was set at 25.0 ± 0.1 °C. Experiments were carried out in a light-tight flat-bottom glass cell of 10 mm diameter. The temperature was adjusted to the desired values ± 0.1 °C using a Thermo Mix Broaun thermostat (Biotechnology Int.)

2.4. Fluorescence measurements

Steady-state fluorescence spectra were recorded on a Perkin Elmer, LS-50 spectrofluorimeter instrument. The excitation and emission monochromators were set at 370 and 470 nm, respectively. A spectral bandwidth of 3 nm was used. All experiments were carried out using freshly prepared solutions containing 5.0×10^{-4} to 2.5×10^{-3} mol L^{-1} of Flu in acetonitrile, using a 10-mm quartz cuvett.

3. Results and discussion

The development of the chemiluminescent glucose technique was divided into two stages. The goal of the first stage was to establish and optimize the quantitative aspects of the CL reaction in the presence of Flu. For this study, the immobilized enzyme was absent from the system and hydrogen peroxide standards were introduced into the detection cell.

3.1. Selection of the chemiluminescent system

In preliminary experiments, it was found that the addition of few drops of the stock solution of hydrogen peroxide to an acetonitrile or ethylacetate solution containing TCPO and Flu in the presence of suitable catalyst such as sodium salicylate results in a very intense blue light. The sensitized PO-CL spectrum of Flu together with its fluorescence excitation and emission spectrums is shown in Fig. 1.

The intensity of the PO-CL emission is proportional to the initial concentration of the CL reactants [57,58]. Thus to achieve maximum sensitivity, the concentration of TCPO, sodium salicylate and Flu should be optimized.

The first parameter that was investigated is the solvent system which should be used to dissolve TCPO and Flu. In addition the solvent should be water mixable because glucose determination is important in aqueous solutions. Based on these factors, acetonitrile was chosen as the proper solvent.

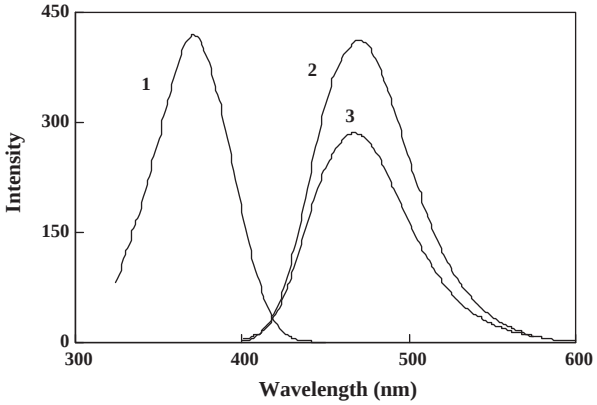


Fig. 1. Fluorescence excitation (1), emission (2) and chemiluminescence (3) spectra of the Flu.

3.2. Optimization of CL reagents

The influence of sodium salicylate concentration on the PO-CL of Flu was studied at constant concentrations of Flu (1.25×10^{-4} mol L^{-1}), TCPO (5.00×10^{-5} mol L^{-1}) and H_2O_2 (1.00×10^{-5} mol L^{-1}) (Fig. 2). The PO-CL intensity found to increase intensely in the presence of sodium salicylate, this effect may be due to catalytic behavior of the salt on the PO-CL system studied [59].

However, at a sodium salicylate concentration upper than 1.00×10^{-6} mol L^{-1} revealed a gradual decrease in the CL intensity and total yield. This is most probably due to the quenching effect of the base at higher concentrations, which begins to decompose the reactive intermediate(s), dioxetane species, and hence reduces the PO-CL light [40,41].

Fig. 3 shows typical response for the PO-CL system of Flu (2.80×10^{-4} mol L^{-1}), sodium salicylate (1.00×10^{-6} mol L^{-1}), H_2O_2 (1.00×10^{-5} mol L^{-1}) in the presence of varying concentrations of TCPO. As it is obvious from the Fig. 3, there is linear correlation between the chemiluminescence intensity and the TCPO concentration up to 7.00×10^{-4} mol L^{-1}. The basis for such linear correlation has already been discussed in literature [30].

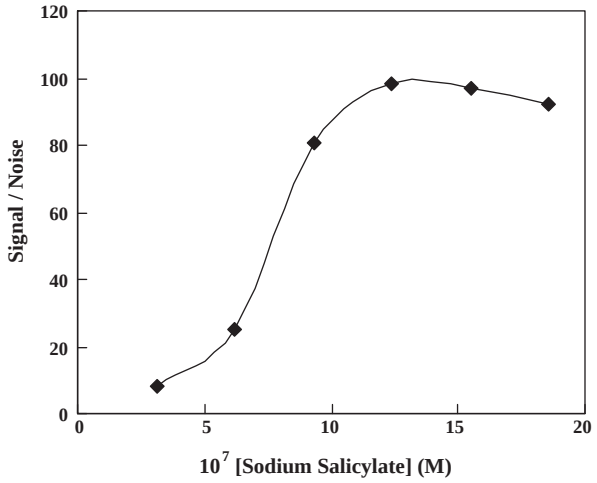


Fig. 2. Effect of sodium salicylate concentration on the CL intensity of TCPO–H_2O_2–Flu system. Conditions: ([H_2O_2] = 1.00×10^{-5} mol L^{-1}, [TCPO] = 5.0×10^{-5} mol L^{-1}, [Flu] = 1.25×10^{-4} mol L^{-1}).

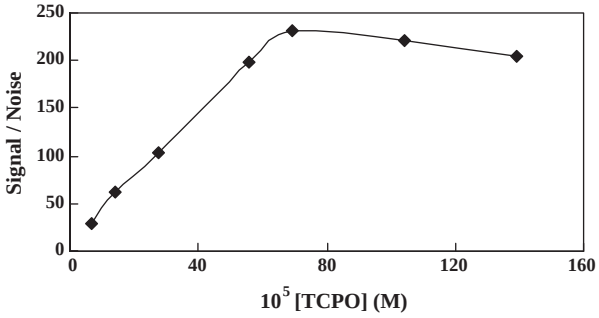


Fig. 3. Effect of TCPO concentration on the CL intensity of TCPO–H_2O_2–sodium salicylate–Flu system. Conditions: ([H_2O_2] = 1.00×10^{-5} mol L^{-1}, [sodium salicylate] = 1.0×10^{-6} mol L^{-1}, [Flu] = 2.80×10^{-4} mol L^{-1}).

However, at concentrations of TCPO above 7.00×10^{-4} mol L^{-1} the increase in signal is no longer proportional to the increase in TCPO concentration and a small decrease in the signal/noise ratio is achieved. Therefore, a concentration of 7.00×10^{-4} mol L^{-1} was used through this study.

The influence of Flu concentration on the PO-CL of the system was studied at constant concentrations of H_2O_2 (1.00×10^{-5} mol L^{-1}), TCPO (7.00×10^{-4} mol L^{-1}) and sodium salicylate (1.00×10^{-6} mol L^{-1}) (Fig. 4). As it has been clearly shown before [40], there is an increase in chemiluminescence of the H_2O_2–TCPO–sodium salicylate–Flu system with increasing concentration of the Flu up to 1.90×10^{-4} mol L^{-1}.

The influence of H_2O_2 concentration on the PO-CL of Flu was studied at constant concentrations of Flu (1.90×10^{-4} mol L^{-1}), TCPO (7.00×10^{-4} mol L^{-1}) and sodium salicylate (1.00×10^{-6} mol L^{-1}) (Fig. 5). It was found that there is a direct linear relationship between the concentration of hydrogen peroxide and PO-CL intensity of the system, at the concentration ranges of 6.25×10^{-7}–1.70×10^{-4} mol L^{-1}. At upper H_2O_2 concentrations the photomultiplier tube detector was saturated. This saturation determines upper limit of glucose concentration that could be measured by the proposed PO-CL system.

With conditions optimized for TCPO-Flu-sodium salicylate system the lower limit of detection was 2×10^{-7} mol L^{-1} peroxide at a signal-to-noise ratio of 3. The lower limit of detection is imposed by background light emission. The reproducibility in peak height was determined by measuring ten replicates of a 5×10^{-6} mol L^{-1} peroxide sample; the relative standard deviation was ±0.8%

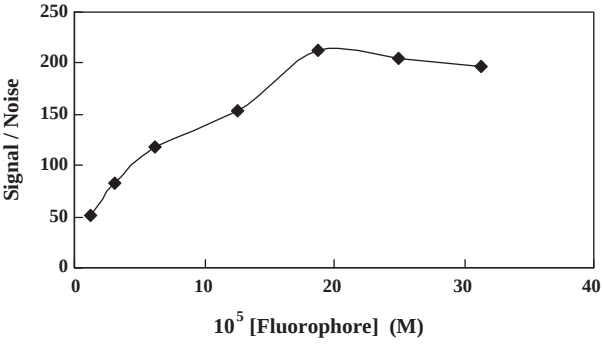


Fig. 4. Effect of Flu concentration on the CL intensity of TCPO–H_2O_2–sodium salicylate–Flu system. Conditions: ([H_2O_2] = 1.00×10^{-5} mol L^{-1}, [sodium salicylate = 1.0×10^{-6} mol L^{-1}, [TCPO] = 7.00×10^{-4} mol L^{-1}).

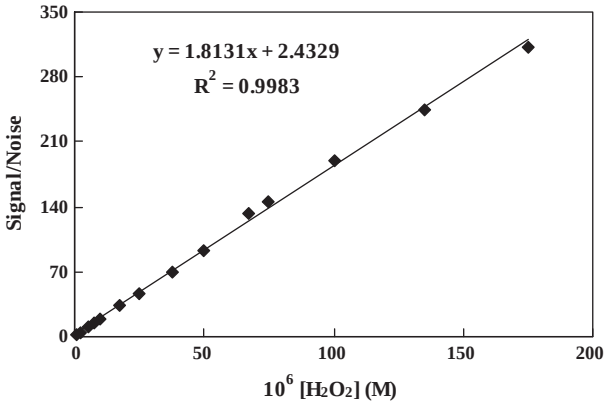


Fig. 5. Calibration curve for H_2O_2 determination with TCPO–H_2O_2–sodium salicylate–Flu system.
Conditions: ([Flu] = 1.90×10^{-4} mol L^{-1}, [sodium salicylate] = 1.0×10^{-6} mol L^{-1}, [TCPO] = 7.00×10^{-4} mol L^{-1}).

3.3. Catalytic oxidation of glucose at the Nafion/GOD/GNPs/CNTs graphite biosensor

Once the parameters for the chemiluminescent determination of peroxide were optimized, the effective conditions on the catalytic oxidation of glucose must be optimized. Thus the GOD–CNTs–GNPs-nafion film was fixed into a suitable glass reactor designed for catalytic oxidation of glucose. Then, 250 μL of glucose of an appropriate concentration (in 0.001 mol L^{-1} phosphate buffer pH 7) was transferred into the glass reactor. The cell was closed and the contents were stirred for 8 min; then stirring was stopped and 150 μL of the resulting solution was injected to the CL measurement cuvette containing following reagents: 50 μL of TCPO (7×10^{-4} mol L^{-1} in acetonitrile), 10 μL of sodium salicylate (5×10^{-4} mol L^{-1} in acetonitrile) and 50 μL Flu (5×10^{-4} mol L^{-1} in acetonitrile). The cuvette was quickly shaken for 2 s and the maximum CL intensity was recorded. The maximum intensity was also achieved in 4–10 s.

Kinetic study on the PO-CL reaction between TCPO and H_2O_2 in the presence of Flu has been completed in our laboratory and the results will be published in the next.

3.4. Effect of CNTs and GNPs

The effects of CNTs and GNPs on the GOD/nafion film behavior were studied and the results are shown in Fig. 6.

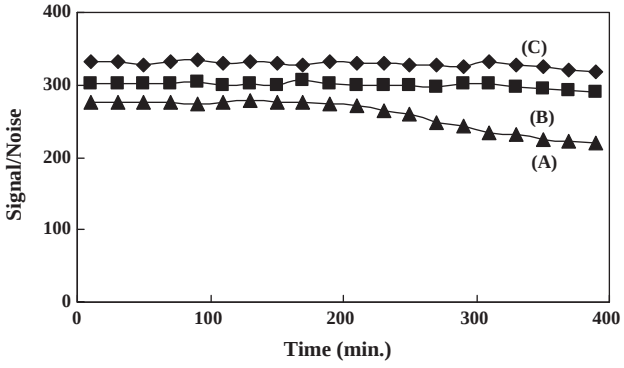


Fig. 6. Effect of CNTs and GNPs on biosensor behavior, (A) GOD/nafion/graphite, (B) GOD/CNTs/nafion/graphite and (C) GOD/GNPs/CNTs/nafion/graphite, Conditions: ([Flu] = 1.90×10^{-4} mol L^{-1}, [sodium salicylate] = 1.0×10^{-6} mol L^{-1}, [TCPO] = 7.00×10^{-4} mol L^{-1}, [glucose] = 1×10^{-4} mol L^{-1}).

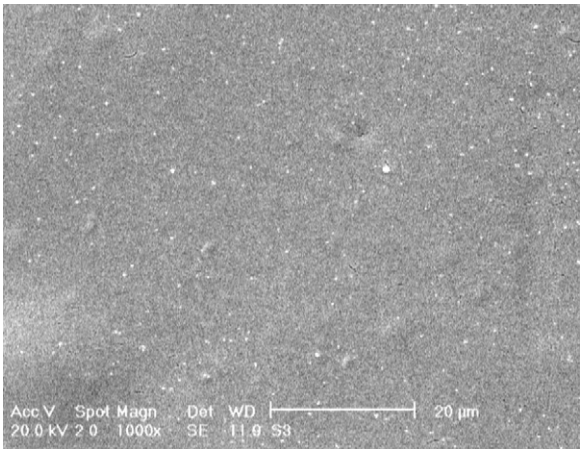


Fig. 7. SEM image of GOD/GNPs/CNTs/nafion film on graphite support.

Data in A, B and C series obtained with GOD/nafion, GOD/CNTs/nafion and GOD/CNTs/GNPs/nafion film respectively. As it is obvious from Fig. 6, presence of the CNTs and GNPs in the nafion film not only enhances the signal/noise ratio but also improves the stability of the nafion film response. These effects are good evidence for effective adsorption of enzymes onto GNPs and CNTs which can act as tiny conduction centers and can improve the enzymes stability in the nafion film [43,60].

As shown by scanning electron microscopy (SEM) (Fig. 7) films formed from GOD/CNTs/GNPs/nafion are uniform. Nafion assists the dispersion of CNTs and GNPs, whereby the CNTs and GNPs remain well dispersed on prolonged standing. Thus, all measurements were performed by GOD/CNTs/GNPs/nafion composite biosensor.

3.5. Effect of pH

The pH of the buffer solution is an important parameter that affects the CL intensity and hence has to be optimized if very sensitive assay is required. The effect of pH on the CL intensity was investigated using 0.001 mol L^{-1} phosphate buffer in the pH range of 5.5–9 (Fig. 8). The optimum pH was obtained at about pH 7.0. It can also be concluded that the CL intensity decreases slightly at pH values higher than 8. Due to the nature of the glucose oxidase reaction with glucose, the pH of the buffer was maintained at pH 7.0 where the enzymatic reaction is optimum. Further study showed

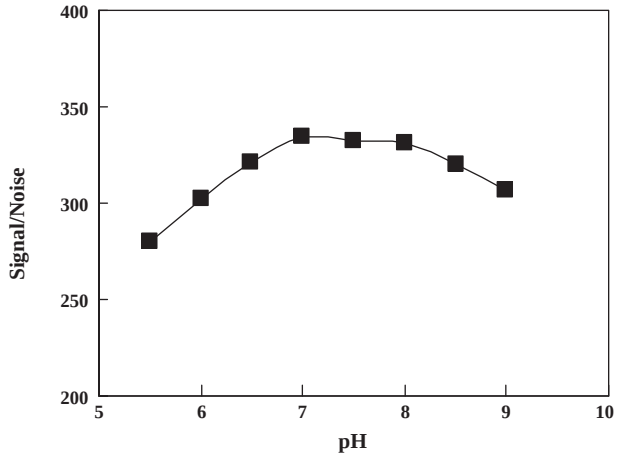


Fig. 8. Effect of pH on signal/noise ratio, conditions: ([Flu] = 1.90×10^{-4} mol L^{-1}, [sodium salicylate] = 1.0×10^{-6} mol L^{-1}, [TCPO] = 7.00×10^{-4} mol L^{-1}, [glucose] = 1.20×10^{-4} mol L^{-1}).

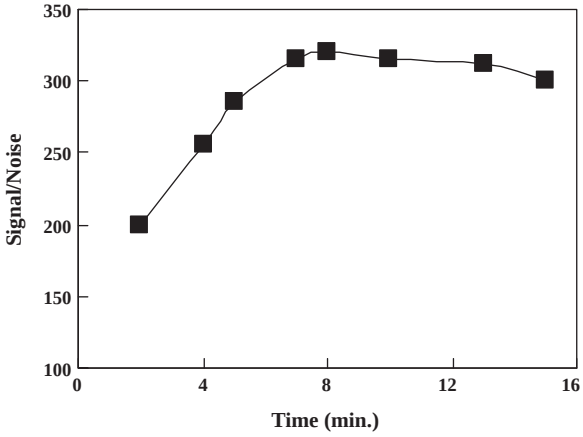


Fig. 9. Effect of action time of enzyme on signal/noise ratio. Conditions: ([Flu] = 1.90×10^{-4} mol L^{-1}, [sodium salicylate] = 1.0×10^{-6} mol L^{-1}, [TCPO] = 7.00×10^{-4} mol L^{-1}, [glucose] = 1.00×10^{-4} mol L^{-1}).

that the best precision was achieved at this pH, and consequently all measurements were performed at pH 7.0.

3.6. Effect of action time of enzyme

The action time of enzyme is considered as the time interval between the injection moment of the glucose solution into reaction cell in the presence of GOD/CNTs/GNPs/nafion composite biosensor, and the moment of starting the injection of resulting solution into CL cell. In order to reach the optimum action time, under the same experimental conditions, the CL measurements were carried out at different time intervals in the range of 0–15 min (Fig. 9). The results clearly revealed that 8 min is the optimum action time.

3.7. Stability, reproducibility and calibration curve of biosensor

Stability is a basis requirement of glucose biosensors. In general, the decrease of activity of enzyme-based biosensors was caused by leaching and inactivation of enzyme. The inactivation of enzyme is mainly caused by unsuitable temperature, and the leaching of enzyme depends on the absorbent structure. The stability of nafion/GOD/GNPs/CNTs graphite biosensor was examined with 8×10^{-5} mol L^{-1} glucose (Fig. 6). The data showed that the sensitivity of nafion/GOD/GNPs/CNTs graphite biosensor remained constant over the first 8 h, and then, it decreased to approximately 80% of the original value, and remained almost constant up to 10 days.

With conditions optimized for the GOD-PO-CL system, the linear range for glucose was 2.25×10^{-6} to 1.75×10^{-4} mol L^{-1} (Fig. 10). The limit of detection (LOD) based on 3σ of the blank was 1.00×10^{-6} mol L^{-1}. The reproducibility in peak height was determined by measuring ten replicates of a 2.5×10^{-5} mol L^{-1} glucose sample; the relative standard deviation was $\pm$ 1.2%.

3.8. Interference studies

Glucose is an important component in biological and food samples. Some components of those samples that possibly interfered with glucose determination were tested. Glucose (10^{-5} mol L^{-1}) was placed in the reaction cell and increasing amounts of different compounds were added into the solution. A material was considered not to interfere if it caused a relative error <5% during the measurement of 10^{-5} mol L^{-1} glucose solution. The results are depicted in Table 1. It was found that at least 15 times of sucrose, fructose, lactose and 150 times of uric acid and ascorbic acid has no

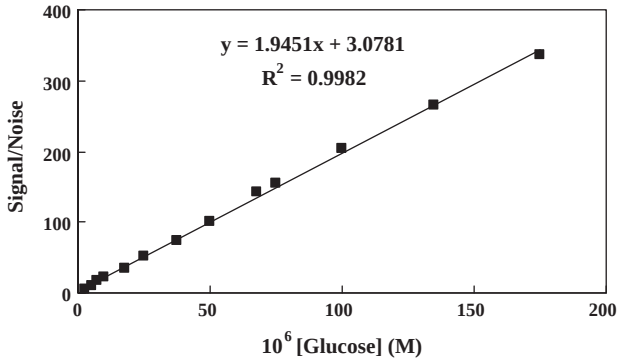


Fig. 10. Calibration curve for glucose determination with nafion/GOD/GNPs/CNTs graphite biosensor Conditions: ([Flu] = 1.90×10^{-4} mol L^{-1}, [sodium salicylate] = 1.0×10^{-6} mol L^{-1}, [TCPO] = 7.00×10^{-4} mol L^{-1}).

Table 1
Tolerable concentration of interferences on glucose determination.

Interference	Tolerable concentration (mol L^{-1})
Sucrose	3.75×10^{-4}
Fructose	4.00×10^{-4}
Lactose	1.50×10^{-4}
Glutamate	9.25×10^{-3}
Uric acid	8.50×10^{-3}
Ascorbic acid	3.75×10^{-3}

Table 2
Determination of glucose in serum and urine samples.

Sample no.	Blood serum (mg dL^{-1})		Urine (mg dL^{-1})	
	CL biosensor	Photometric	CL biosensor	Photometric[a]
1	112.5 ± 3.2	114	17.7 ± 0.5	6
2	105.7 ± 1.3	106	15.4 ± 0.3	6
3	89.8 ± 2.3	85	4.3 ± 0.2	–
4	88.4 ± 2.1	91	5.0 ± 0.1	–
5	94.3 ± 1.8	93	2.8 ± 0.1	–

[a] LOD of the Pars Azmoon photometric kit is 5 mg dL^{-1}.

interference with the determination of glucose. Since at the chosen pH, many species such as uric acid, ascorbic acid are anions, nafion film will block off this anions. In addition PO-CL reaction is free of uric acid interference [34–37].

3.9. Application of biosensor for determination of glucose in human serum and urine samples

In order to examine the applicability of the glucose biosensor, we measured the glucose concentration in the real samples. Blood and urine samples from different persons were supplied by the Imam Khomeini Hospital (Tehran, Iran). Plasma and urine samples were analyzed with photometric kits (Pars Azmoon, Iran) in the hospital. The comparative results are shown in Table 2. These results indicate that better sensitivities can be obtained with the GOD-PO-CL system.

4. Conclusion

A simple and high sensitive biosensor for detection of glucose based on immobilization of glucose oxidase/CNTs/GNPs in nafion film at the surface of graphite was demonstrated. Coupling the sensitivity of the chemiluminescent systems with the selectivity of the enzymatic reactions offers several advantages over standard methods for glucose analysis. Because glucose oxidase is immobilized, the chemiluminescent method has the economical advantage of reusing the enzyme for many samples. Instrumentation requirements for the chemiluminescent method are minimal. Since excited Flu molecules are the only emitting species, only a photomultiplier, without a monochromator, is required. Because the chemiluminescence system is free of uric acid interference the method let us quantitative determination of urine glucose.

Based on the principle of this CL biosensor, it can be assumed that a series of CL enzyme sensor can be designed for detection of those substrates that can react with their corresponding enzymes to produce hydrogen peroxide. As the sensitivity of chemiluminescence permits glucose analysis of sample sizes as small as 10 μL, the method may be applied to both routine clinical analysis and research.

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