Iranian Journal of Analytical Chemistry
Volume 5, Issue 1, March 2018 (9-16)

مجله ایرانی شیمی تجزیه
دوره ۵، شماره ۱، فروردین ماه ۱۳۹۷ (۱۶-۹)

تعیین گزینشی گلوکز در پلاسمای خون با استفاده از حسگر زیستی آمپرومتری گلوکز مبتنی بر آنزیم گلوکز اکسیداز و فیلم پیچیده‌ی متشکل از کیتوسان/ نافیون/ مایع یونی/ فروسن

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تاریخ دریافت: ۲ اسفند ۱۳۹۶ تاریخ پذیرش: ۲۳ فروردین ۱۳۹۷

Selective Determination of Glucose in Blood Plasma by Using an Amperometric Glucose Biosensor Based on Glucose Oxidase and a Chitosan/ Nafion/ IL/Ferrocene Composite Film

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Received: 21 February 2018 **Accepted:** 12 April 2018

چکیده

نوعی از حسگر زیستی آنزیمی مبتنی بر وساطت فروسن به منظور اندازه گیری گلوکز به عنوان یک آنالیت مهم زیستی تهیه شده است. مخلوط همگنی از فروسن شامل کیتوسان (CS)، نافیون (Nf) و یک مایع یونی مبتنی بر ایمیدازولیوم ۱- بوتیل- ۳ متیل ایمیدازولیوم هگزا فلوئورو فسفات [BMIM]HF_6PO_4، تهیه گردید. مخلوط مذکور بر سطح الکترود کربن سرامیک قرار داده شد و به منظور پایداری مکانیکی حسگر زیستی لایه ای از آنزیم بین دو لایه از این مخلوط به دام انداخته شد. برای بررسی عملکرد تجزیه ای حسگر زیستی از ولتامتری چرخه ای و کرونو آمپرومتری استفاده شد. با اعمال پتانسیل ثابتی (۱۸۰ میلی ولت) بر پلاسمای انسانی برای مدت زمان کوتاهی (۱۰۰ دقیقه)، کاهش قابل ملاحظه ای در علایم مزاحم ناشی از آسکوربیک اسید (AA) و اوریک اسید (UA) ایجاد شد. مقادیر پارامتر های عملی مانند حساسیت (۲,۷۳μA $mM^{-1}cm^{-2}$)، محدوده ی خطی (۹۵,۲۳-۱۳۶۷,۵ μM) و حد تشخیص (۴۸μM) با مطالعات کرونو آمپرومتری به دست آمدند. هم چنین ثابت ظاهری مایکلیس منتن K_m (۳,۵۲ mM)، محاسبه شد. برای تخمین صحت و دقت عملی حسگر زیستی، آزمایش بازیابی گلوکز وارد شده در پلاسمای طبیعی انجام شد.

واژه‌های کلیدی

گلوکز اکسیداز؛ اکترود کربن سرامیک؛ حسگر زیستی؛ مایع یونی؛ کیتوسان؛ نافیون.

Abstract

A kind of ferrocene mediated enzymatic biosensor was fabricated in order to the measurement of glucose as an important biological analyte. A homogenous mixture of ferrocene including Chitosan (CS), Nafion (Nf) and an imidazolium based ionic liquid, 1-butyl-3-methyl imidazoliumhexafluorophosphate, [BMIM]HF6PO4, was prepared. The mentioned mixture was cast on the surface of carbon ceramic electrode and an enzymatic layer was entrapped between two layers of this mixture in order to improving the mechanical stability of the biosensor. Cyclic voltammetry and chronoamperometry were used to investigate the analytical performance of the biosensor. Remarkable deduction of interfering signals produced by AA and UA was obtained by applying a constant potential (180mV) on the human plasma for a short period of time (100min). Values of practical parameters such as sensitivity (2.73 μA $mM^{-1}cm^{-2}$), linear range (95.23–1367.5 μM) and detection limit (48 μM) were obtained by the chronoamperometric studies. The apparent Michaels-Menten constant, Km (3.52 mM) was also calculated. In order to estimate the practical accuracy and precision of the enzymatic biosensor, a test of spiked glucose solution recovery in natural plasma was done.

Keywords

Glucose Oxidase; Carbon Ceramic Electrode; Biosensor; Ionic Liquid; Chitosan; Nafion.

1. INTRODUCTION

Nowadays glucose is introduced as one of the most noteworthy clinical analytes [1]. Chemically modified electrodes (CMEs) have been used as suitable instruments for fabrication of enzymatic biosensors [2-8]. On the other hand, there are some basal factors which must be noticed in fabrication of these kinds of biosensors including: easy preparation, rapid, accurate and selective analytical response, preservation of enzyme biological activity in the modifying matrix, creation an efficient electrical communication

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between the redox center of an enzyme and the electrode and etc [9]. Sol–gel-derived carbon ceramic composite electrodes (CCEs), showed proper features which commuted them to appropriate supports for the enzymatic biosensors [10]. Some of these features are simple preparation method, convenience in immobilizing ferrocene species and biomolecules, chemical inertness, mechanical stability and high biocompatibility [11-14].

Ferrocene and its derivatives have attracted much attention in the development of the second generation (mediated) enzymatic biosensors due to their excellent redox properties [15-19]. In spite of all efficiencies of the redox couple of Fc+/Fc it shows an undeniable deficiency and that is their leakage of the electrode surface into the electrolyte solution [20].

To prevent this phenomenon, we are perforced to use some materials, which cause more adhesion and stability for the mediated biosensors. Chitosan and Nafion are two familiar polymers displaying plenty of desirable properties and thereupon they can be used as the most promising materials for enzyme immobilization [21]. Applications of room temperature ionic liquids (RTILs) in electrochemistry were reported by many research groups [22]. Negligible vapor pressure and good solubility besides their biocompatibility makes them appropriate materials for the fabrication of enzymatic biosensors and it is proved that an enzyme can preserve its own activity and stability by usage of a suitable ionic liquid [23-29].

In this manuscript, an enzymatic glucose biosensor was constructed with the aid of Nafion, Chitosan, Fc, an imidazolium based RTIL and glucose oxidase enzyme as the biological receptor. The mixture of these materials was immobilized on the surface of CCE. Electrochemical responses of the designed biosensor to glucose and the other extant oxidizable biological interferences (AA and UA) were investigated in human plasma as the real sample.

2. EXPERIMENTAL

2.1. Chemicals and reagents

CS (85%), Nafion (5% in ethanol), GO_x (from Aspergillus niger, E.C. 1.1.3.4), Ferrocene(Fc) and 1-butyl-3methylimidazoliumhexafluorophosphate [BMIM] HF_6PO_4), were obtained from Aldrich Chemical Company. Other chemicals were of analytical grade and they were used without further purification. Methyltrimethoxysilane (MTMOS) and Fine-powdered graphite purchased from Merck company were used to prepare the sol-gel solution. $Na_2HPO_4$was used to prepare a phosphate buffer solution (pH 7.0, 0.1M). All of the solutions were prepared by double distilled water.

2.2. Apparatus

The electrochemical experiments were accomplished by an AUTOLAB PGSTAT-100 (potentiostat/galvanostat) equipped with a USB electrochemical interface and driven by a GPES 4.9 software package (Eco Chemie, The Netherlands) in conjunction with a three-electrode system and a personal computer for data storage and processing. A three-electrode cell system including a saturated calomel electrode(SCE) as the reference electrode, a platinum wire as the auxiliary electrode, and the enzymatic modified CCEs (geometric surface area of 0.119 cm^2) as the working electrode was employed for the electrochemical studies. All experiments were performed at room temperature.

The bare carbon ceramic electrodes were prepared according to the following procedure described by the Lev and co-workers [30]. 0.6 ml MTMOS, 0.9 ml methanol and 0.6 ml HCl 0.5 M catalyst were mixed for 20 min, then 0.3 g graphite powder was added and the obtained mixture was shaken for additional 10 min. A piece of Teflon tube of 1 cm length and approximately 4 mm inner diameter was filled with the sol–gel carbon mixture and the mixture was then dried overnight under ambient conditions (25ºC). The electrode was polished with polishing paper and subsequently rinsed with doubly distilled water. Electric contact was made with copper wire through the back of the electrode. To fabricate the enzymatic biosensor following processes should be done respectively: a) A Fc–composite solution including 120 µl IL 1% in ethanol, 20 µl Nf 1% in ethanol and 0.0026 g Fc was prepared. Then a 140 µl of CS solution (10 mg/ml CS in acetic acid 1%) was added to the former solution. The last mixture was placed in Ultra sonication for 15 min to become homogenous. b) A 10 µl of the obtained mixture was coated on the clean and rinsed surface of the bare CCE and the electrode was left to be dried at 25ºC (Fc-composite/CCE). c) GO_x solution was obtained by solving 0.002 g GO_x in 200 µl phosphate buffer solution (0.1 M, pH =7.0) and 10 µl of the mentioned solution was cast on the electrode surface (GO_x/Fc-composite/CCE). This electrode was left at 4ºC to be dried. d) At the last step 3 µl of the homogenous Fc-composite solution prepared at the first step, was coated on the enzymatic biosensor and again it was left at 4ºC to be dried absolutely.

3. RESULTS AND DISCUSSION

3.1. Electrochemical properties of the Fc-composite/CCE

Fig. 1. Indicates cyclic voltammograms of the modified CCE with the Fc homogenous mixture (composed of Fc, CS, Nf and IL) in a phosphate

buffer solution (0.1 M, pH =7.0) at a scan rate of 100 mvs^{-1}. 5, 10 and 15 µl of the mentioned mixture was coated on the surface of the bare CCE, respectively. In spite of larger anodic peak current indicated by the modified CCE with 5µl of the mixture, the volume of 10 µl was selected as the suitable one due to less film leakage of the electrode surface and more stability in this volume. Further increase in peak currents was not seen in the case of volume 15 µl. typical cyclic voltammograms for modified electrode showed a pair of reversible redox peaks with low peak potentials. The anodic and cathodic peak potentials were located at 0.202 and 0.132 V, respectively.

3.2.Electrochemical properties of the GOx/Fc-composite/CCE

Fig. 2. Exhibits cyclic voltammograms of the modified electrode before and after enzyme immobilization in a phosphate buffer solution (0.1 M, pH =7.0) at a scan rate of 100 mvs^{-1}. It is obviously observed that a light increase is produced in the height of both anodic and cathodic peak currents after enzyme casting showing the improvement of electron transfer and conduction effects affiliated redox center in the GO_x enzyme on the electrode surface.

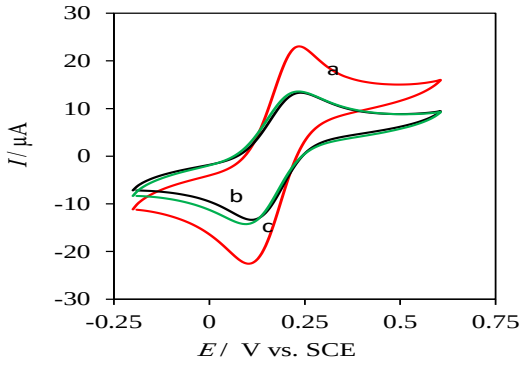

Fig. 1. Cyclic voltammgrams of Fc-CS-Nf-IL/CCE with 5 µl (a), 10 µl (b) and 15 µl (c) of Fc-composite in phosphate buffer solution (0.1 M, pH =7.0) at 100 mVs^{-1}.

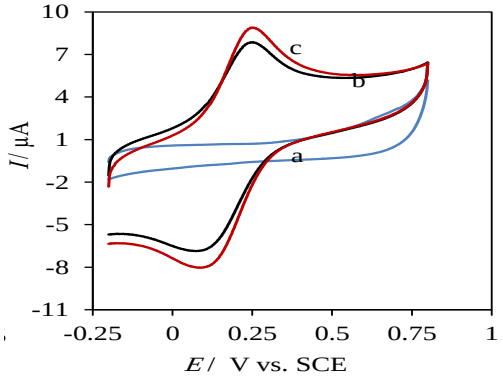

Fig. 2. Cyclic voltammograms of a) bare CCE ,b) Fc-composite/CCE and c) GO_x/Fc-composite/CCE in phosphate buffer solution (0.1 M, pH =7.0) at 100 mVs^{-1}.

3.3.Electrochemical response of the enzymatic biosensor to glucose

Fig . 3A. Exhibits the cyclic voltammograms of the bare CCE and the enzymatic biosensor in absence and presence of 5 mM glucose in a phosphate buffer solution (0.1 M, pH =7.0) at a scan rate of 20 mvs^{-1}. According to the obtained plots, an adequate response is attained toward 5 mM glucose. Also Fig. 3B. shows voltammetric responses of the enzymatic biosensor to continuous addition of glucose concentrations (0 – 45 mM) at the same circumstance.

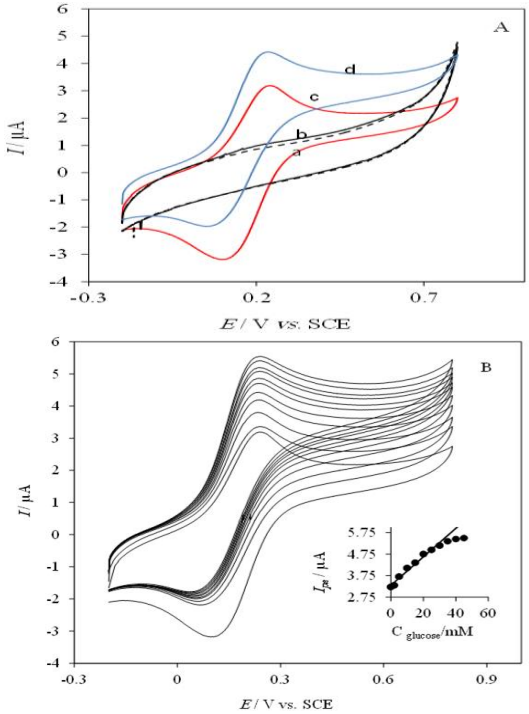

Fig. 3. (A). Cyclic voltammograms of bare CCE in 0 mM glucose (a), 5 mM glucose (b), GO_x/Fc-composite /CCE in 0 mM glucose (c) and in 5 mM glucose (d) in phosphate buffer solution (0.1 M, pH =7.0) at 20 mVs^{-1}. (B) Cyclic voltammograms of GO_x/Fc-composite /CCE in phosphate buffer solution (0.1 M, pH =7.0) containing different concentration of glucose (0, 2, 5, 10, 15, 20, 25, 30, 35, 40 and45 mM), at 20 mVs^{-1}. Inset: plot of catalytic peak current versus glucose concentration scan rate: 20 mVs^{-1}.

The optimized experimental results show that the peak current rises sharply with the increasing concentrations of glucose and a maximum response is obtained at the concentration of 40 mM. Moreover, the effect of varying the scan rate on the performance of the enzymatic biosensor was also studied and the results were shown in Fig. 4. With an increasing scan rate, the CV peak currents of the GO_x/Fc-composite /CCE increased in the scan rate range from 10 to 600mVs^{-1}, but peak potentials were nearly independent of the scan rate, indicating that the mediator was efficiently connected on CCE for facile charge transfer. It can

also be observed that the both of anodic and cathodic peak currents increase linearly with the scan rate in the range of 10 to 200 mVs^{-1} proving surface controlled characteristic of charge transfer process and in higher scan rates it increases linearly with the square root of potential scan rate showing that electrode process is controlled by mass transfer.

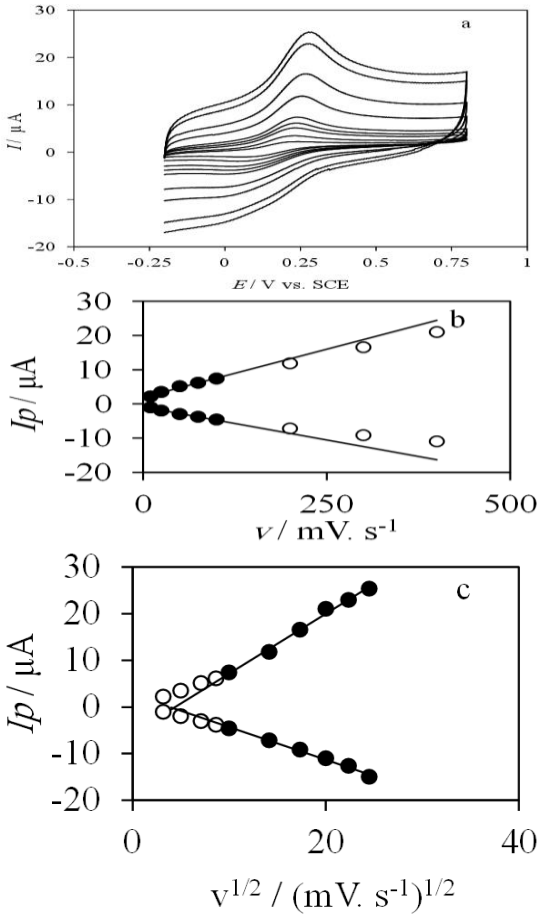

Fig. 4. (a) Cyclic voltammograms of GO_x/Fc-composite/CCE in 5 mM glucose at different scan rates (1-10): 10, 25, 50, 75,100, 200, 300, 400, 500 and 600 mVs^{-1} in phosphate buffer solution (0.1 M, pH =7.0).
(b) plot of anodic peak currents versus scan rates.
(c) plot of anodic peak currents versus square root of scan rates.

3.4.Effect of mediator presence on the response of the biosensor toward glucose

In order to investigate the effect of ferrocene existence on the analytical response of biosensor to 5 mM glucose, two enzymatic modified electrodes were prepared as follows: the first electrode was modified with the mixture including IL, CS and Nf and GOx (GO_x/ CS-IL-Nf/CCE) and the second one was modified with the mixture including IL, CS, Nf and Fc and GO_x (GO_x/CS-IL-Fc-NF/CCE). Voltammetric responses of prepared biosensors to glucose were compared with each other. A comparable increase is observed in the analytical response to glucose by the second biosensor in the same circumstance which is referred to the distinct mediating effect of Fc+/Fc (Fig.5). 3 µl of the Fc-composite solution was coated on the enzymatic layer in the last step of biosensor construction due to the prevention of enzyme leakage from the electrode surface. In fact, an enzymatic layer is entrapped between two layers of the Fc composite and therefore the stability of the biosensor was enhanced. More amount of Fc composite (5µl) blocked the paths of electron transfer along the modifying film.

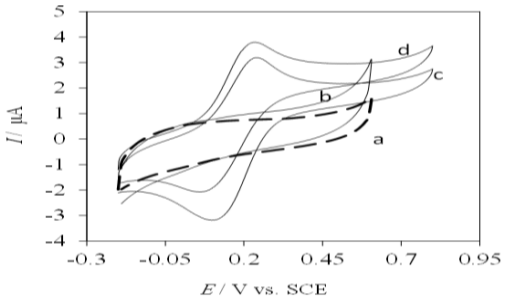

Fig. 5. Cyclic voltammgrams of bare CCE in 0 mM glucose (a), GO_x/CS-Nf-IL/CCE in 5 mM glucose (b), GO_x /Fc-composite / CCE in 0 mM glucose (c) and GO_x/Fc-composite/CCE in 5 mM glucose in phosphate buffer solution (0.1 M, pH =7.0) at scan rate $20mvs^{-1}$.

3.5.Effect of loaded enzyme volume and variations of pH and temperature solution on the response of the enzymatic biosensor

To evaluate the optimized volume of the enzyme, various values of GO_x solution (3, 5, 10, 15 and 20 µl) were cast on the modified electrode with Fc-composite, respectively. Catalytic responses of the biosensor to glucose increases linearly with the volume of coated enzyme from 3 to 10 µl and it reduces by a further increment of enzyme loading (15 and 20µl) pointing the insulation effect of protein barrier around the redox center of the enzyme (Fig.6A.).Temperature and pH are two effective factors on the performance and the nature of GO_x enzyme. To investigate the effect of temperature on performance of the designed enzymatic biosensor, cyclic voltammograms of the biosensor were obtained in 5 mM glucose solution in a phosphate buffer solution (0.1 M, pH =7.0) at a scan rate of $20mvs^{-1}$ at different temperatures (5-59 ºC). According to the resultant curves, anodic currents rise with increasing temperature in the range of 5 to 51 ºC and a significant decrease of an analytical response of biosensor is observed at higher temperatures (56 and 59 ºC) referring denaturation of the susceptible protein structure of the GO_x enzyme.(Fig. 6b). Equation 6-1 refers to the dependence of biological activity of the GO_x enzyme to pH variations [31].

$$GOD - FAD + 2e^- + 2H^+ \leftrightarrow GOD - FADH_2 (Eq.6-1)$$

According to this equation proton shortage, reduces the bio catalytic activity of the GO_x enzyme. On the other hand, ionic properties of the incorporated Chitosan and Nafion change with pH variations due to their structural characteristics. However, the activity of chitosan reduces in basic solutions and the Nafion's enhances in such solutions. With considering all these aspects, it can be derived: that values of anodic peak currents, referred to electron transmittals along the immobilized modifying film, increase with pH rising in the range of 4 to 7, regularly and the slight response increase in pH of 8 is due to Nafion ionization in basic solutions and in further values of pH the current tends to be almost fixed (Fig.6c).

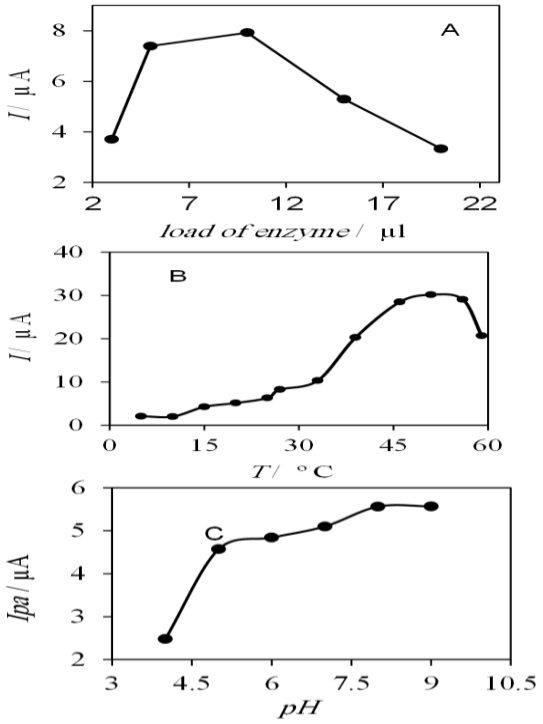

Fig. 6. Plot of oxidation peak currents of GO_x/Fc-composite /CCE versus enzyme loading (A), temperature (5, 10, 15, 20, 25, 27, 33, 39, 46, 51, 56 and 59 ˚C) (B) and pH (C) in 5 mM glucose solution.

3.6. Amperometric detection of glucose at the enzymatic biosensor

Fig.7. shows the typical current- time responses at the enzymatic biosensor for successive addition of glucose (1-21 mM) in a phosphate buffer solution (pH 7.0, 0.1M) at an operational potential of 300mV. As it is expected for simple enzymatic reactions: the rate of enzymatic reaction increases proportionlately with the glucose concentration in the range of low amounts of substrate and it tends to be fixed in high substrate concentrations due to saturation of active sites of the enzyme (Michaels – Menten mechanism). By attention to this concept and using of Lineweaver- Burk equation value of apparent Michaels – Menten constant was calculated 3.25 mM [32]. This value seems to be a desirable concentration for this biosensor. The amperometric response increases linearly in the range of 95.23× 10^{-6}- 1367.5×10^{-6}M. The linear equation was Ip=0.3552 C+2.9292 with a correlation coefficient of 0.9988. The calculated detection limit was 48 ×10^{-6} M which was obtained from the signal to noise ratio of 3 (S/N=3). The sensitivity of the biosensor was calculated 2.98 µA $mM^{-1}cm^{-2}$ (with a surface area of 0.119 cm^2 for the bare CCE) (Fig.8). The storage stability of the sensor was found to be 4 weeks stored at 4 ˚C under dry condition with Only a small decrease with a relative standard deviation of 2/9%.

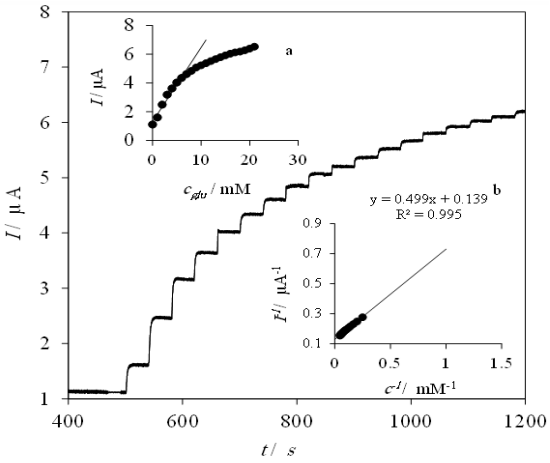

Fig. 7. Typical amperometric response of GO_x/Fc-composite /CCE on the successive addition of 1 mM glucose to a stirring phosphate buffer solution (0.1 M, pH =7.0). Applied potential=300 mV. Insets: (a) Calibration plot of concentration of glucose (1-21mM) versus current (b) I^{-1} versus C^{-1}.

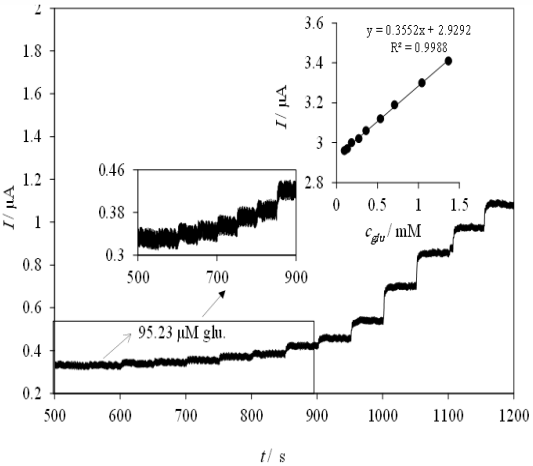

Fig. 8. Chronoamperometric current response of GOx/ Fc- composite/CCE to increasing concentrations of glucose at 300 mV in phosphate buffer solutions (0.1 M, pH =7.0). Inset: plot of current response versus glucose concentration.

3.7.Interferences study

Ascorbic acid and Uric acid are the most familiar and persecutor biological interferences during glucose detection. Nafion membrane coating on the electrode surface is a suggested method in plenty of researches due to its protection characteristics[33]. The mentioned method is significantly efficient in case of in vivo detections. In this work, Due to the operating potential (300mV), the biosensor is sensitive to electroactive interfering species in human blood such as ascorbic acid, and uric acid. A remarkable deduction of interfering signals caused by AA and UA was created by removing them from the experimental solution and natural sample (human plasma) via applying a constant potential of 180mV on a 5mM AA solution for a short time of 37 min. At this potential, AA and UA will be changed into their stable oxidized forms which do not disturb the glucose detection. This act was done on a 5 mM AA and 5 mM glucose solution, respectively. Resultant achievements exhibited that applying the constant potential, had no effects on the glucose analytical signal (Fig.9A). Response of enzymatic biosensor to 5 mM glucose was obtained in presence of 0.1 mM of interfering species (Fig.9B). On the other hand, Fc mediated enzymatic biosensors are scarcely used as in vivo detection instruments due to their toxicity, So this method can be a promising method for removing interferences for in vitro biological detections. The sensor was insensitive to sucrose and fructose and acetaminophen.

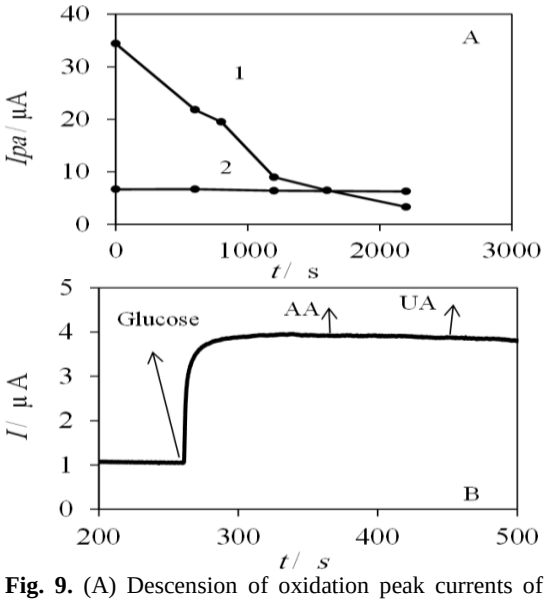

Fig. 9. (A) Descension of oxidation peak currents of GO_x/Fc-CS-Nf-IL/ CCE with time of applying constant potential of 180 mV for 37 min on 5mM AA (1) and 5mM glucose solution (2). (B) Electrode response to glucose (5 mM) and interfering compounds (0.1mM) at 300 mV.

3.8.Application of the biosensor for determination of glucose in human plasma

Plasma is a complicated matrix, including 91.5% water, 7% protein, 1.5 % salts, sugars, and lipids. Concentration of AA, UA and glucose in this matrix are: 0.023-0085, 0.15 - 0.48 and 4.4-7.2 mM respectively. In this work, the constant potential was applied to absolute human plasma for a time of 100 min and it obviously showed desirable descensions in oxidation peak current value proving the efficacy of the suggested method for removing interference species (Fig.10). Concentration of existence glucose in human plasma was obtained 7.02 mM by standard addition technique which is in the range of normal range. To evaluate the accuracy and practical precision of enzymatic biosensor in amperometric detection, a test of recovery of spiked glucose solution in plasma was done. Table .1. reports the obtained data from the determination of glucose human plasma.

Table 1. Determination of glucose in human plasma.

Spiked Glucose in Plasma (mM)	Found Glucose (mM)	Recovery (%)
0	7.02	-----
1	7.099	97
2	8.96	97.3
3	9.99	99

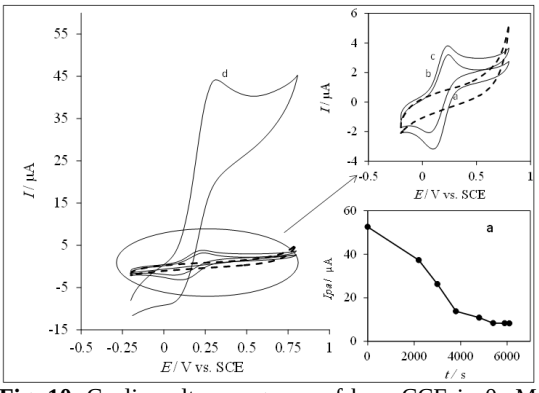

Fig. 10. Cyclic voltammograms of bare CCE in 0mM glucose (a) and GO_x/FC-CS-Nf-IL/CCE in 0mM glucose (b), in 5 mM glucose (c) in phosphate buffer solution (0.1 M, pH =7.0) and in absolute human plasma (d) at scan rate 20mVs^{-1}. inset a: plot of reducing peak currents with time of applying constant potential (0, 37, 50, 63, 80, 90, 98 and 100 min) on absolute human plasma.

4. CONCLUSION

A Ferrocene mediated enzymatic glucose biosensor was successfully prepared by immobilization the GO_x-layer between two layers of Fc – composite solution. By assembling the beneficial characteristics of CS and Nf and the conduction effects of Fc and an imidazolium based RTIL, an acceptable selectivity and mechanical

stability have been shown for the fabricated biosensor. Applying a constant potential of 180mV on human plasma solution for a short time of 100 min was used as a novel method for removing of coexisted interferences in the biological sample. Desirable accuracy and precision were observed by a test of recovery of the spiked glucose solution in human plasma.

ACKNOWLEDGMENTS

The authors are grateful to Research Office of the Azerbaijan University of Tarbiat Moallem for financial support.

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