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# Fabrication of Nafion/Silver Nanoparticles/Reduced Graphene Nanosheets /Glucose Oxidase Nanobiocomposite for Electrochemical Glucose Biosensing

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**Abstract**- The aim of this work was fabrication of a nanobiocomposite as a smart biosensor for monitoring glucose. A glucose nanobiosensor based on silver nanoparticles (SNs) and reduced graphene nanosheets (rGNs) composite was successfully fabricated and developed. The SNs-rGNs nanocomposites were successfully prepared and coated on the surface of strip. The reduced graphene nanosheets (rGNs) support incorporated to zero-dimensional Ag nanoparticles arrayed in the third dimension amended a glucose biosensor with superior sensing characteristics. The sensor abilities were certified by changing sensor performance (i.e., sensitivity, limit of detection, and linear range) through using a novel nanocomposite. This approach to fabricate a biosensing device qualifies a high sensitive biosensor design that demonstrated outstanding biological sensing performance such as boosted glucose sensitivity (32 nM limit of detection, 14.1 $mAM^{-1}cm^{-2}$ sensitivity, 200 nM to 9 mM linear sensing range), a long stable shelf-life (>21 days), and a high selectivity. To the best our knowledge, this device is the first biosensor protocol for glucose monitoring.

**Keywords**- Biosensor, Silver nanoparticles, Reduced graphene oxide, Glucose oxidase, Nafion

## 1. INTRODUCTION

The exquisite and rapid determination of glucose concentration in physiological systems is interesting due to its importance in living organisms, especially in those suffering diabetes mellitus [1]. Several different technologies, approaches and methods were developed to measure blood glucose dose, such as fluorimetry [2-5], HPLC [6,7], electrochemistry [8-10] and etc. The comparison among these technologies reveals commercial electrochemical biosensors based on enzyme modified electrodes have demonstrated enormous benefits, advantages and applications owing to several advantages including simple electrochemical techniques, redox systems and the sensitivity, specificity and selectivity of the catalytic redox enzymes. Electrochemical nanobiosensors have been broadly explored, amended and developed in clinical detection of analytes. These items can be due to their wonderful advantages comprising high sensitivity, great selectivity, fast response time and suitable linearity [10].

Recently, carbon based nanostructures have been increasingly utilized to fabricate various devices, such as batteries [11,12], supercapacitors [13-15], transistors [16], solar cells [17], sensors [18-22] and hydrogen storage [23], especially in biosensors due to their individual physicochemical properties (high surface area, excellent conductivity, high mechanical strength, ease of functionalization, mass production, low cost, good electron transfer kinetics and biocompatibility) [24]. In recent times, graphene nanomaterials have also been incorporated into biosensors [25] and biofuel cells [26]. Inherent graphene is a zero-gap semiconductor which has exceptionally high electron mobility in ambient, that is even higher than carbon nanotubes [24]. The preparation of high-quality 2 dimensional carbon nanosheets is the first and most critical step, as the existence of residual defects will heavily impact the electronic properties of graphene [27].

In recent years, silver nanoparticles (AgNPs) have attracted increasing research attention for applications in industry and medicine. It is well known that AgNPs is the best conductor among metals, so Ag nanoparticles may facilitate more efficient electron transfer than gold nanoparticles in biosensors. AgNPs generally possess excellent catalytic activity and offer a hospitable environment for biomolecules [28].

In previous study a novel biosensor with excellent sensitivity and bioactivity was developed for PKU monitoring with a nanocomposite, rGO-AgNPs-nafion. Here, Ag nanaoparticles and 2-D rGO were used to promote the sensitivity and output responses of a glucose sensor. The value in use of AgNPs incorporated reduced graphene nanosheets (rGO) to develop an electrochemical biosensor with remarkable sensitivity to determine glucose was investigated. To the best our knowledge, the combining the rGO-AgNPs composite and nafion for glucose biosensors applications has been unexplored till now. A sturdy sensor biofunctionalization protocol was applied to electrodeposit biocomposite with an e conductive material nafion onto the rGNs-AgNPs nanocomposite surface. The versatility of

the glucose biosensor has been studied by cyclic voltammetry (CV) in presence ferricyanide and chronoamperometry (CA). The selectivity capabilities of the biodevice also have been investigated and discussed. The stability of the sensor also optimized. The developed biosensor presents to be worthy with high glucose sensitivity even after 3 weeks of use, minimal interference from typical electroactive species (i.e., dopamine, glycine, l-cysteine, ascorbic acid and ethanol) typically found in human serum samples, and a low limit of detection (LOD) and wide linear sensing range that considerably improves upon some features of glucose biosensors previously reported in the literature. To the best our knowledge, this device is the first biosensor protocol for glucose determination.

## 2. EXPERIMENTAL

### 2.1. Reagents and apparatus

The reduced graphene oxide synthesized from graphene-based nanosheets via chemical reduction of exfoliated graphite oxide according to literature [29]. Nafion and glucose were supplied from Merck co. Glucose oxidase (GOD) was purchase from Sigma co. $K_3[Fe(CN)_6]$ and other chemicals were of analytical grade.

Evaluating the electrochemical tests were carried out using a conventional three electrode cell interfaced to PalmSense (type 3). A tow-line silver ink covered onto PET sheets was applied as the working and reference electrodes, respectively. All sensor tests were carried out using an electrochemical cell of 10 µl at ambient conditions.

### 2.2. Biosensor design

*2.2.1. Preparing the rGNS-AgNPs-nafion composite electrode*

Reduced graphene oxide (rGO) sheets were synthesized by reduction of graphite oxides (GOs) with hydrazine hydrate according to literature [30]. Briefly, the GOs were firstly produced by oxidation of graphite with $H_2SO_4$ and potassium permanganate, which were gradually dropt within 0.5 h under vigorous stirring in an ice bath. Then, the reaction mixture was heated to 35 $^oC$ for 45 min with vigorous stirring, and then the GOs were obtained by adding hydrogen peroxide into the reaction mixture, and in turn treated ultrasonically for 30 min. The rGO sheets were prepared by the reduction of GOs with hydrazine monohydrate at 95 $^oC$ for 1 h. The product was washed with deionized water several times to remove excess hydrazine, and finally dried under reduced pressure for 24 h. A tow-line thin film of silver ink was coated onto a polyterephthalate (PET) wafer at a base pressure of $5.0\times10^{-7}$ Torr. Fig. 1 represents the procedure to fabricate the developed glucose biosensor. The electrodes was pretreated in 1.0 M NaOH solution and the potential of working electrode was held at +1.0 V for 5 min. rGNS suspension was prepared by dispersing 10 mg of rGNS and 4 mg of AgNPs

in 10 mL ethanol with ultrasonic agitation for about 2 h. Then, 50 µL of 50% nafion and 50 µL of 3.2 $mg.mL^{-1}$ rGNs and Ag solution were blended under continuous stirring. The obtained solution was applied to treat the working electrode as follows: 5 µL of the prepared solution was casted onto the working electrode surface (one line of the strip) and allowed to be dried at room temperature for at least 5 h. In an effort to improve the electroactive nature of the rGNs and AgNPs based composite electrodes were exposed to an $O_2$ plasma etch within a Plasma Tech Reactive Ion Etch (RIE).

*2.2.2. Enzyme immobilization*

The protocol for immobilization of biocatalysts is of importance due to avoiding instability and inactivity of the enzyme electrodes. Before electrodeposition of the enzyme solution onto the rGNS-AgNPs surface, a composite of GOD and nafion was prepared. First, a solution of GOD ($5mg.dL^{-1}$) was added to nafion solution. Next, GOD/nafion solution was electrodeposited onto each rGNs-AgNPs-nafion electrodes via constant current pulses of 1.05 mA that were applied between the working electrode (rGNs-AgNPs-nafion) and auxiliary electrode (Pt wire) for 500 cycles. In order to avoid the denaturing GOD, the bioelectrode were kept at 4 $^oC$ for 24 h.

## 3. RESULTS AND DISCUSSION

### 3.1. Fabrication of nanobiocomposite based GOD-nafion/AgNPs-rGNS-nafion

The main approach to fabricate the glucose biosensor is simple and inexpensive showing in the Fig. 1. A nanofilm contains rGNS-AgNPs nanocomposite was prepared and dispersed in nafion solution. Then, dip coating technique was used to fabricate a suitable substrate for immobilization of GOD. Fig. 2a represented the morphology of nanocomposite coated on the strip. As shown in the Fig. 2a, a smooth surface of nanostructures dispersed in nafion was established. Finally, a mixture of GOD and nafion was provided and immobilized onto the activated nanocomposite surface utilizing physicochemical bonds for constructing a biological activated biosensor (Fig. 2b). Fig. 2b depicted enzyme morphology immobilized on the nanocomposite surface. The results of Fig. 2b demonstrated that a distinct homogeneous layer has been formed on the nanocomposite. Although physical attachments are weak, these bonds retain catalytic activity of the redox enzymes. Contrariwise, in spite of robust strength of chemical bonds, these attachments reduce enzyme catalytic activity. Therefore, combining the physical and chemical attachments can integrate both advantages.

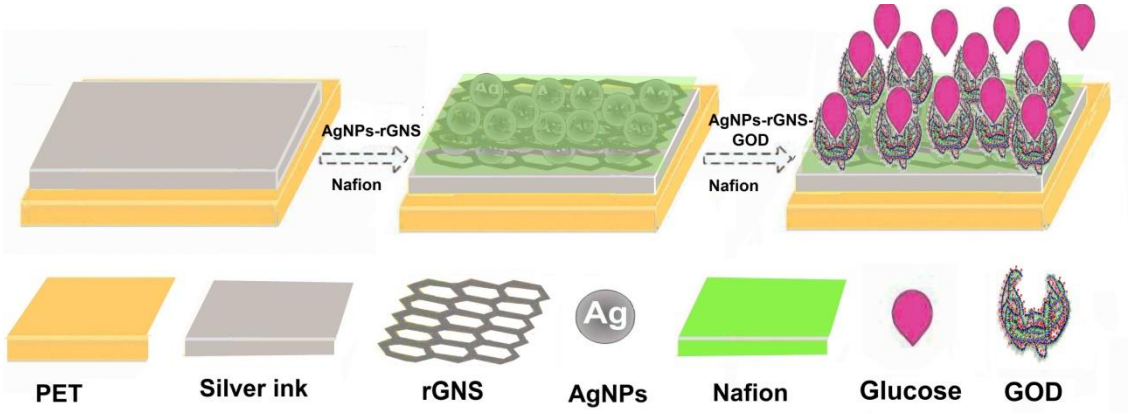


**Fig. 1.** Schematic representation of preparing the rGNS-AgNPs-nafion/GOD- nafion

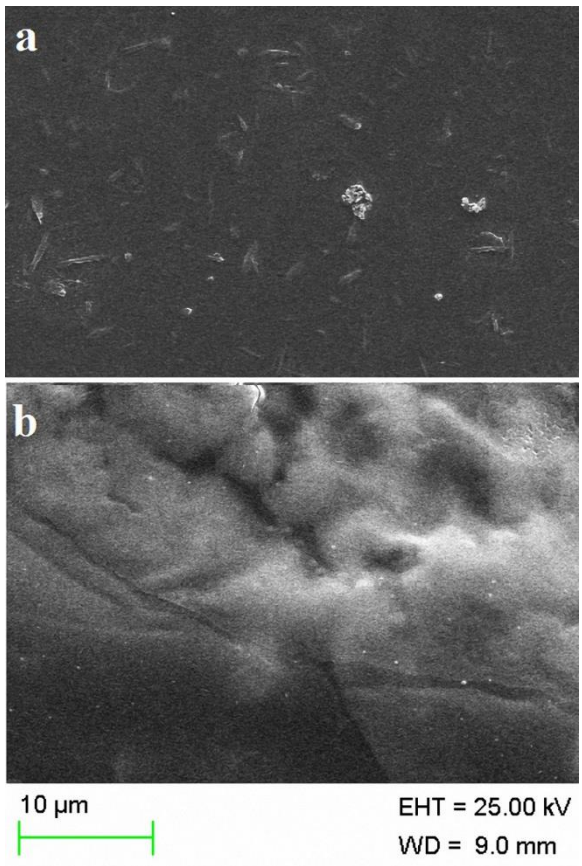


**Fig. 2.** SEM micrograph of a) rGNS-AgNPs-nafion coated on the strip and b) GOD-nafion immobilized on the rGNS-AgNPs-nafion nanocomposite

### 3.2. Effect of pH value

Evaluating the influence of pH on the current response of the enzyme electrode is a critical step in the design and construction of biosensors since the biological catalytic behavior of the GOD immobilized onto active surface is pH dependent. The performance appraisals of pH quantity related to the current response of the enzyme based biosensor were

conducted in a series of phosphate buffer solution (PBS) with the pH from 4.5 to 8.5 in glucose solution (0.1 mM). The best results demonstrated that maximum value of pH for this device is 7 (data not shown). Therefore, pH 7 was chosen for the all electrochemical tests and detection of glucose.

### 3.3. Electrochemical characteristics of the modified electrode

The first step in evaluating electrochemical biosensor is cyclic voltammetry (CV) of the ferricyanide based electrodes as an available and useable method to evaluate the electroanalytical behavior of the surface activated with modified nanomaterials.

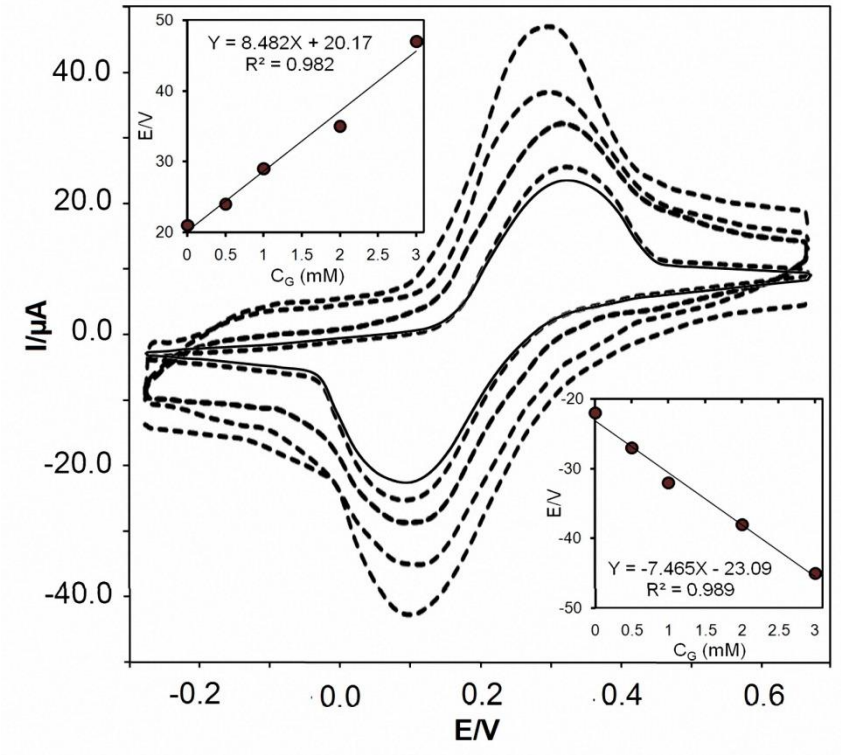


**Fig. 3.** CVs of bare rGNS-AgNPs-nafion/GOD-nafion composite strip (line), successive addition of glucose to cell (dashed) in 5 mM $[Fe(CN)_6]^{4-/3-}$, Scan rate: 50 mV/s

Fig. 3 shows the CVs evaluation of the enzyme modified bioactivated electrode during stepwise increment of glucose in 5 mM $K_3Fe(CN)_6$ with a scan rate of 50 mV/s. It can be observed in Fig. 3, there is a well-defined oxidation and reduction peak of ferricyanide based material at the nanocomposite electrode (line). Upper diagram depicted the nanocomposite amended electrode in presence of 0.5 mM glucose. As can be seen in this curve, the peak current enhanced significantly, displaying that enzymatic reaction could raise the electron transfer rate. In the sense that, when a mass (glucose) are adsorbed onto active site of the enzyme electrode, the response current increases sharply (dashed). Therefore, the nanocomposite has greatly facilitated the electron transfer between the analyte and the

glucose biosensor. The CV evaluation of GOD immobilized on the enzyme modified electrode was investigated in the buffer, PBS (pH 7). The rGNS-AgNPs-nafion/GOD-nafion based nanocomposite modified electrode behaved as a super conductive composite so GOD immobilized on nanocomposite was applied to fabricate a highly sensitive biosensor for diabetes diagnosis. As can be seen in Fig. 3, there is a significant increment in the oxidation current at 320 mV *vs*. Ag/AgCl, proving that in this potential the rGNS-AgNPs dispersed into the nafion based film is worthwhile for transferring electrons between the materials and the active site of the enzyme (redox center). For instance, with altering the glucose concentration level from 0.5 to 3 mM in PBS (pH 7), a linear relationship between the change of the reduction peak current of GOD and the glucose concentration can be observed ($R^2$=0.982 for oxidation, $R^2$=0.989 for reduction; Fig. 3). This relationship demonstrated that the biosensor follows Randles–Sevcik equation so it is suitable for glucose biosensing.

One of the most critical issues for estimating a biosensor is choosing a suitable and reliable electrochemical technique. In this study, several electrochemical techniques were investigated and amperometry was finally selected because of its high accuracy and excellent sensitivity. Therefore, the steady-state current obtained from amperometry technique was utilized to evaluate electrochemical performance of the biosensor with increasing the concentration of glucose. Fig. 4 depicts a sample current-time followed amprometry response of the rGNS-AgNPs-nafion/GOD-nafion/GCE based strip on regular increment of glucose level under the optimized ambient conditions. It is obvious in Fig. 4, increasing the concentration of the analyte, glucose, causes enhancing the current response of the biosensor. It is clear from inset of Fig. 4, a privileged linear proportion between the output current and glucose concentration can be observed (from 200 nM to 9 mM with a correlation coefficient of 0.996). The linear regression equation of the sensor output was i(μA)=1.652C(glucose, mM)+1.511. The dynamic amperometry plot elaborated an extraordinary sensitivity equal to 14.1 $mAM^{-1}cm^{-2}$ and a limit of detection (LOD) equal to 32 nM (signal-to-noise ratio=3). The obtained strip achieved 98.5% of the steady-state current response within 7 s. The output current response obtained amperometry inclined to achieve a saturation point at uppermost analyte concentration, proving excellent characteristics of the Michaelis–Menten kinetics (data not shown). The value of $K_m$ was calculated as 4.23 mM according to Lineweaver–Burk equation indicating that the immobilized GOD on the modified nanocomposite based electrode possessed higher enzymatic catalytic bioactivity.

Moreover, the results of the developed glucose biosensor have been compared with some the best other glucose biosensors in the literature. The comparative results revealed the developed biosensor showed outstanding performance in terms of linearity range, LOD and output response time. The utilization of rGNS-AgNPs-nafion/GOD-nafion nanobiocomposites could dramatically increase the capability of biocatalyst immobilization,

which caused diffusing the glycoprotein shell of the GOD and gained access to the active catalytic sites of the enzyme. In the next experiment, direct electron transfer between redox site of the enzyme and electrode surface was observed, which could efficiently reduce current response time, increase linearity range and amplify the sensitivity.

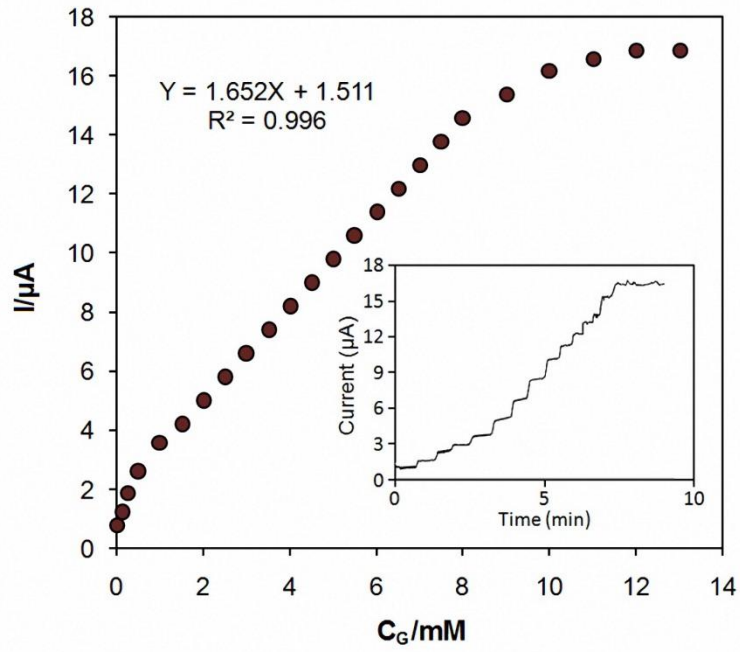


**Fig. 4.** Amperometric response of the glucose biosensor in stirred solutions to the successive additions of glucose in 0.1 PBS (pH 7.0); the inset exhibits linear calibration curves.

### 3.4. Shelf-life, Selectivity and Reproducibility

There are many materials and species in human fluids (such as ascorbic acid, l-cysteine) that can be caused adverse reactions. This issue decreases accuracy and selectivity of the enzyme strip to determination of analyte. Therefore, investigating the effect of possible interfering species on the detection of glucose is hypersensitive. Here, interferent effects were analyzed using 3.0 mM glucose, dopamine, glycine, l-cysteine, ascorbic acid and ethanol, causing side effects from 3.86% (max) to 1.64% (min) in maximum reducing or increasing the output current response of the glucose strip, respectively (Fig. 5). As a result, this device suggested that the biocatalytic biodevice has high selectivity and excellent specificity due to the catalytic enzyme reaction of GOD. Therefore, the selectivity evaluations demonstrated that above five interferents resulted in a so negligible decrease or increase in the output current signal, suggesting that the direct electron transfer between redox of the enzyme, GOD, and strip surfaces of the biosensor helped to eschew the intermediate reactions of

interferences. The reproducibility of a biodevice is a critical key to evaluation of a reliable device in clinical applications.

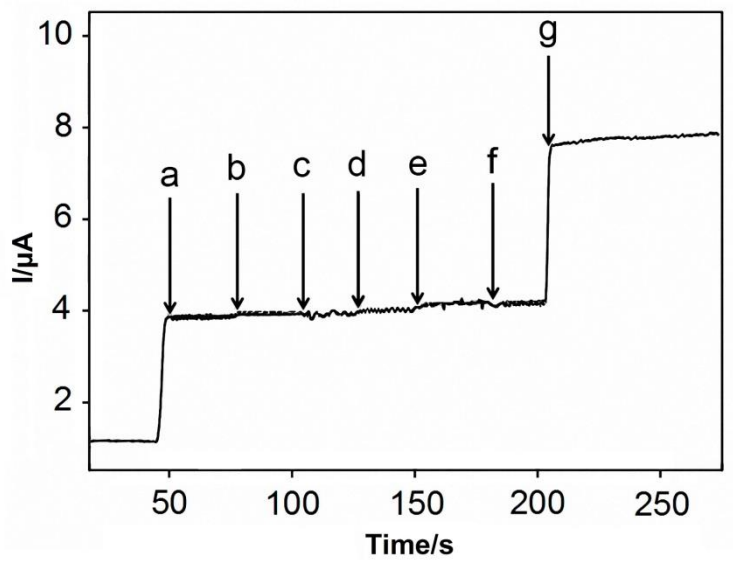


**Fig. 5.** Selectivity experiments of the enzyme electrode in presence of interferents: (a) 1 mM glucose; (b) 1 mM ethanol; (c) 1 mM glycin; (d) 1 mM l-l-cysteine; (e) 1 mM ascorbic acid; (f) 1 mM dopamine; (g) 3 mM glucose

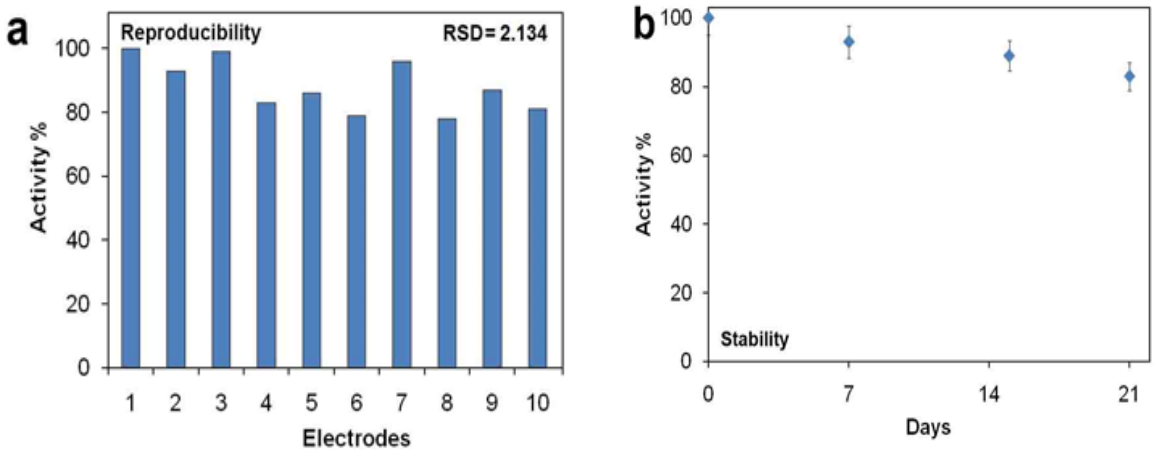


**Fig. 6.** (a) Evaluation of ten different electrodes fabricated in the same conditions on current response to 200 µM glucose; (b) Stability of the enzyme-functionalized electrode after 21 days in 2 mM glucose concentration. Initial working volume: 10 µl; supporting electrode: 0.1 M PBS buffer pH 7 (N=5)

Biosensor reproducibility was perused via comparing the output current of several different bioelectrodes under similar conditions. Ten different bioelectrodes were selected to evaluate reproducibility of the sensor (Fig. 6a). Fig. 6a shows the output currents of ten

enzyme electrodes provided a relative standard deviation (RSD) value of 2.134%, which elaborated that the rGNS-AgNPs-nafion/GOD-nafion based biosensor had remarkable reproducibility. One of the challenging issues in the performance of the enzyme base biosensors is shelf-life of the enzyme electrodes due to low stability or instability of biocatalysts after a few days. The amended bioelectrode retains more than 83.4% of its accuracy and sensitivity even after 21 days. Stability experiments demonstrated that the sturdy nature of the enzyme immobilization protocol with amperometry testing and storage (Fig. 6b).

### 3.5. Human real sample analysis

In addition to interference study it seems that the human real sample analysis is necessary for deep evaluation of a diagnostic device. Therefore, the potential of the developed electrochemical glucose biosensor for determining glucose in human fluids was investigated due to further certification. The device was utilized to determine glucose level in human blood by the gold standard addition method. As we know the normal range of glucose level in human blood are $4.5<C_{glucose}<6.5$. The human blood samples offered by several humans were gained from Tehran University of Medical Sciences. Ten real extractions (from different humans) with different glucose concentrations of 0.5, 1.0, 3.0, and 8.0 mM (evaluated with a gold standard method) was tested by the device. As can be seen in Table 1, the recovery of the output current responses of the enzyme strip is in the range of 93.91–103.02% and the relative standard deviation (RSD %) is in the range of 1.29–4.3%.

**Table 1.** Determination and recovery results of glucose in the real samples by the biosensor (N=5)

| Samples | The addition content | The detection content | RSD (%) | Recovery (%) |
|---|---|---|---|---|
| Human blood (200 µM) | 0.0 | 0.206 mM | 3.64 | - |
| | 0.5 | 0.687 mM | 2.45 | 98.14 |
| | 1.0 | 1.067 mM | 4.13 | 88.92 |
| | 3.0 | 3.281 mM | 3.62 | 102.53 |
| | 8.0 | 8.126 mM | 2.09 | 99.1 |

These results depicted that the developed biodevice can be initially applied to monitor diabetes in clinical diagnosis. Furthermore, the broad linear sensing range of the biosensor not only enables sensing within the physiological range for blood glucose of healthy [3.6 mM

and 7.5 mM (65 mg/dL–135 mg/dL)] and diabetic [1.1 mM and 20.8 mM (20 mg/dL–350 mg/dL)] patients; it enables glucose sensing in saliva, tears, and urine as well [31]. By comparing our work with other reported publications in the literature, it can be found that both the recovery and relative standard deviation (RSD) values were suitable for the detection of glucose in human serum samples, which confirmed that the GOD based electrochemical biosensor is able to provide the potential for the determination of target biomolecule, glucose, in real samples. Therefore, this approach for developing the glucose biosensor opens novel and reliable possibilities for new biomaterials for glucose sensors in which glucose levels from human serums could be determined simultaneously.

## 4. CONCLUSION

A high selective and good sensitive electrochemical biosensor with the utilization of rGNS-AgNPs-nafion/GOD-nafion has been proposed for glucose monitoring. The rGNS-AgNPs-nafion composites enhanced the bioactive electrode surface area and served as an excellent support for immobilizing the enzyme, GOD. The final biosensor showed high performance included low detection limit, wide linear range, fast response time and excellent sensitivity. A more controllable, stable and reproducible composite film casted onto strip can be achieved using homogeneous solution of rGNS-AgNPs-nafion witch have an excellent glucose biosensing capability due to the immobilizing the GOD-nafion. These interesting results were related to some factors, comprising the controlled highly sensitive surface area, the low electrical resistance pathway at the rGNS-AgNPs-nafion interface, the selective enzyme adhesion and direct electron transfer that occurred between the GOD and rGNS-AgNPs-nafion interfaces. The fabricated GOD based biosensor exhibited good sensing performance with a linear response up to 9 mM with detection limit of 328 nM and also had excellent selectivity over electroactive species and stability. It may be expected that other materials, biomolecules and mediators can be combine in the rGNS-AgNPs-nafion/GOD-nafion for the fabrication of better biosensors.

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