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ARTICLES

Easy and Low-Cost Chitosan Cryogel-Based Colorimetric Biosensor for Detection of Glucose

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Abstract—A colorimetric biosensor for glucose detection has been studied based on chitosan cryogel supporting material for enzyme immobilization. The detection was based on the glucose conversion to hydrogen peroxide by glucose oxidase, then a titanium(IV) oxysulfate was used to measure hydrogen peroxide, indicated by the formation of yellow color. The color change with the concentration was then recorded by a commercial scanner and analyzed using an ImageJ software. The fabricated biosensor allows to easily prepare by in-tips enzyme immobilization with user-friendly operating using micropipette by the suck-hold-release method for the determination of glucose. Enzyme immobilization has been optimized including the amount of enzyme and the reaction time. The biosensor showed a high operational stability for up to 56 measurements using a single immobilized enzyme, with a wide linear range (0.3 to 3.0 mM glucose), high specificity, and also agreed with the standard method used in hospitals to detect blood glucose (the Wilcoxon signed-rank test, $P > 0.05$).

Keywords: cryogel, glucose biosensor, chitosan, colorimetric

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Early detection and diagnosis require less treatment and provide cost-effectiveness of diseases management. Glucose detection in the blood is the most feasible approach for early detection of diabetes mellitus. Several methods for the determination of blood glucose levels have been reported based on chemical, enzymatic and affinity-based detection principles. Biosensors utilizing biosensing elements to detect glucose showed the advantages of their high selectivity due to the nature of the biosensing, for example, glucose oxidase enzyme, a highly specific substrate for glucose. Many strategies have been reported to improve the glucose biosensor performances, mainly used to increase the sensitivity, selectivity, and stability or to decrease the time and cost of the analysis.

The high stability of the biosensor can be achieved by the maintenance of enzyme activity by the use of natural polymers, such as chitosan [1], agarose [2] and alginate [3] as supporting materials. Chitosan can be prepared in cryogel form, providing high surface area, stability and sensitivity in the biosensor development for glucose [1], sialic acid [4], microalbumin [5] and carcinoembryonic antigen [6] determination. However, the use of electrochemical detection in the previously described chitosan cryogel based biosensors showed some disadvantages, such as the high cost of the instrument, laborious electrode preparation and relative complexity of the operation.

On the other hand, colorimetric sensors have received considerable attention due to their simplicity, high sensitivity and low cost. The rapid growth of digital imaging devices leads to the development of cost-effective methods based on the color change with the analyte concentration with the use of smartphone camera [7], web-cam [8], digital camera [9] and scanner [10] as detectors. The ability of such devices to take digital images have been reported with their functioning as spectrophotometers with the resolution of 5 nm [11] providing a wide range of applications, such as flow immunoassay [12], quantum-dots for labeling bacteria [13] and label-free photonic crystal biosensors [14].

The aim of this work was to develop a simple procedure for the preparation of a low-cost colorimetric glucose biosensor using chitosan cryogel as a supporting material. The proposed biosensor was easy to prepare by growing chitosan cryogel in a micropipette tip and immobilizing the glucose oxidase in the chitosan cryogel with the measurement of the concentration by a commercial document scanner. The biosensor was also easy to operate by a suck-hold-release method

using a micropipette, similar to the daily applied for the handling of liquid samples. This biosensor would be a user-friendly diagnostic and promising detection device, compared to the standard methods for glucose detection.

EXPERIMENTAL

Chemicals and materials. Glucose oxidase (**GOD**) (EC 1.1.3.4, Type II-S, 15–50 units/mg), chitosan from crab shell, titanium(IV) oxysulfate (≥ 29%), glutaraldehyde (Grade II, 25% in H_2O) were from Sigma (Steinheim, Germany). D-(+)-glucose anhydrous, hydrogen peroxide (30%), acetic acid (96%), sodium dihydrogen phosphate monohydrate and di-sodium hydrogen phosphate di-hydrate were obtained from Merck (Germany).

Apparatus and measurements. Scanning electron microscopy (JEOL JSM-6510, Japan) was used to characterize the chitosan cryogel surface. Shimadzu Biospec 1601 UV-Vis spectrophotometer (Shimadzu, Japan) was used to measure the sample color change in the preliminary study. Commercial document scanner (Canon LiDE 120, Vietnam) was used to capture digital images of the samples color changes. An ImageJ software was used to convert digital images into RGB (red, green, blue) intensities. 96 well plates flat bottom (Iwaki, Japan) was used as the sample holder for the reaction and measurements.

Biosensing element preparation. The biosensing element was based on the immobilized glucose oxidase in the chitosan cryogel (Fig. 1). Chitosan solution was prepared by dissolving 2 g in 80 mL of 1% (v/v) of acetic acid, heated with gently stirring and diluted with 1% acetic acid solution to 100 mL. The chitosan solution was then filtered and kept at 4°C. Chitosan cryogel has been prepared by simultaneous polymerization and freezing of chitosan solution using glutaraldehyde as a crosslinker. Chitosan solution of 100 μL was mixed with 5 μL of a glutaraldehyde solution (2.5%, v/v). This mixture was then added to a micropipette tip of 100–1000 μL (blue tip) with a stainless rod as a hollow template. The chitosan polymerization was performed in sub-zero temperature (–20°C) for six hours to allow the cryogelation process to occur. Chitosan polymers were being crosslinked, while water was being frozen allowing porous cryogel backbone to be built. The chitosan cryogel was then thawed at 4°C allowing crystal water to melt, followed by removing from the stainless steel rod to produce the hollow chitosan cryogel (**hChiCryo**) in micropipette tips. The hChiCryo inside a blue micropipette tip was not only easy to prepare, but also had a double function: as a shield of the hChiCryo from physically damage and as enzyme reactor to allow glucose conversion, assisted by micropipette. The chitosan cryogel was then activated using 100 mL of 2.5% (v/v) glutaraldehyde solution for 20 min and rinsed with deionized water. Glucose oxidase (**GOD**) enzyme was prepared in the phosphate buffer solution (50 mM, pH 7.0) with the enzyme concentration of 0.2 U/μL. This enzyme solution of 50 μL was immobilized in the activated hChiCryo for six hours at 4°C. This hChiCryo-GOD was kept at 4°C when not used.

Glucose detection. Glucose detection in the fabricated biosensor was based on the enzymatic reaction of glucose and GOD enzyme. The GOD converts glucose into gluconolactone and hydrogen peroxide. A series of glucose solutions in the concentration range of 0.6–1.4 mM were first tested. The glucose and GOD reaction was performed in the chitosan cryogel inside the micropipette tip, by sucking the glucose solution using the micropipette, holding the glucose solution to allow the enzymatic reaction, continued by releasing the solution to a flat bottom 96 well plate. 50 μL of titanium(IV) oxysulfate solution (2.5%, w/v in 2 M sulfuric acid) was then added to each solution. The well plate was then placed in the document scanner, scanned and converted the RGB intensities using an ImageJ software. The RGB intensities related to the hydrogen peroxide concentration were analyzed. As a comparison, the color change of hydrogen peroxide and an indicator were also analyzed using a spectrophotometer.

Enzymatic optimization. This study was performed similarly to glucose detection with various enzyme modifications. The biosensor optimization included the enzyme amount and the enzymatic reaction time. The enzyme amount studied were 2.5 to 20 U of the GOD enzyme immobilized in the hChiCryo. The optimum amount of the GOD was then used to study the enzymatic reaction time in the range of 0.5 to 10 min. Under the optimal conditions, the fabricated glucose biosensor was used to detect the standard glucose solution from the lowest to the highest concentration to establish the linear range, limit of detection and limit of quantification.

Stability study. One of the advantages of using immobilized enzymes, including the proposed fabricated biosensor, is the reusability. The reusability of the biosensor for glucose detection was studied by measuring 3.0 mM standard glucose solutions continuously. Good stability of the glucose biosensor was determined by the given responses for more than 90% of the initial response.

Selectivity study. Common interferences of glucose detection in blood samples are ascorbic and uric acids [15]. These interferences can result in false positive in some cases, when glucose detection is based on the use of glucose oxidase as a reducing agent, similar to hydrogen peroxide resulting in the conversion of glucose by such enzyme. In this study, the ascorbic acid and uric acid were added to the standard glucose solution. The standard glucose solution of 3.0 mM (normal blood glucose level) was mixed with various concentrations of ascorbic and uric acids.

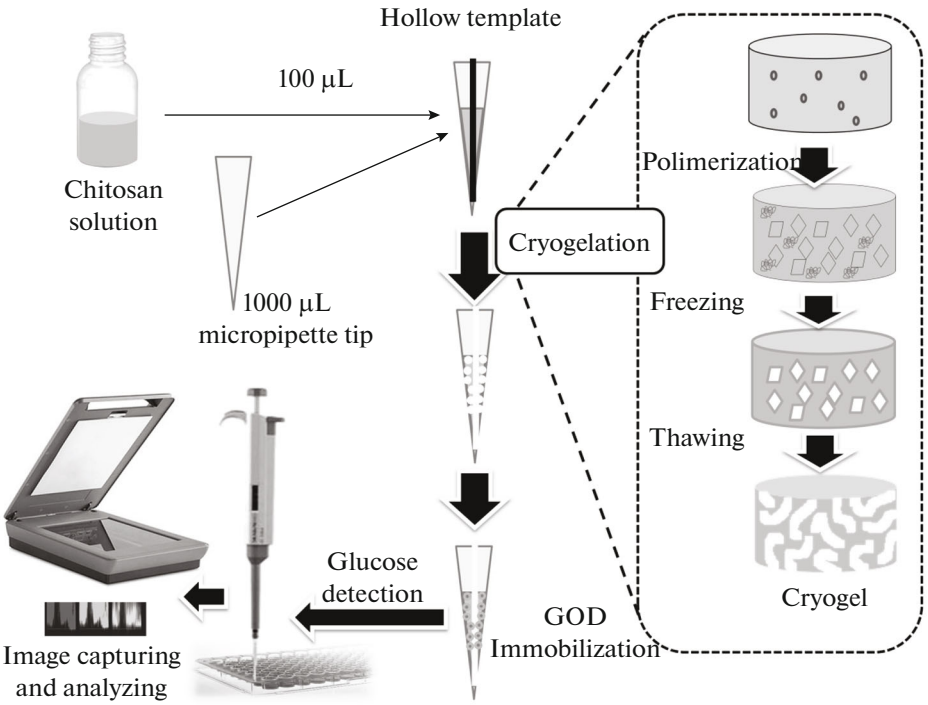

Fig. 1. Hollow chitosan cryogel modified biosensing element preparation. The chitosan cryogel was developed inside a micropipette tips, continued by immobilization of glucose oxidase enzyme.

Real sample analysis. The glucose biosensor performance was tested to determine glucose concentration in the blood sample collected from a local hospital. The glucose level obtained using the proposed biosensor was compared to the glucose concentration in the blood plasma analyzed using a standard hexokinase method (spectrophotometry). Those results were statistically compared using the Wilcoxon signed-rank test [16].

RESULTS AND DISCUSSION

Chitosan cryogel preparation and morphological study. Scanning electron microscopy (**SEM**) images of the fabricated chitosan cryogel showed pore diameters of 10–100 μm (Figs. 2a, 2b). The pore sizes of this chitosan cryogel were slightly larger than the sizes of chitosan-albumin cryogel previously reported, which were in the range of 5–20 μm [1]. It was also confirmed that the non-cryogel chitosan polymerized at 4°C did not show a porous structure (Figs. 2c, 2d).

Glucose detection. This biosensor was designed for user-friendly operating by the method called suck-hold-release. First, glucose solution was sucked using micropipette armed with hChiCryo-GOD tip, and then glucose was being held in the tip acting as the enzyme reactor to allow glucose conversion to occur. Finally, obtained hydrogen peroxide was released from the micropipette tip, followed by its reaction with the indicator according to the scheme presented below. Glucose was first converted to gluconic acid and hydrogen peroxide generated with immobilized glucose oxidase enzyme, Eq. (1), then hydrogen peroxide reacted with titanium(IV) oxysulfate to produce a yellow color of pertitanic acid, Eq. (2).

$$\text{D-Glucose} + O_2 \xrightarrow{\text{Glucose oxydase}} \text{Gluconic acid} + H_2O_2, \quad (1)$$

$$TiOSO_4 + H_2O_2 + 2H_2O \rightarrow H_2TiO_{4(\text{yellow})} + 4H^+ + SO_4^- . \quad (2)$$

The use of titanium sulfate method for hydrogen peroxide detection was selected due to its high speed and formation of a yellow-colored complex stable for at least 6 h [17]. The method was also highly specific to hydrogen peroxide detection without any interference responses [18]. Furthermore, the strongly acidic solution made it pH-independent [17].

This mixture was prepared in the 96-well plate flat bottom to easy capture the color change using a scanner, with its further analyzing using an ImageJ software. Similar software, such as Adobe Photoshop™ (Adobe Inc, Windows-based application) [19] and Color Grab (Loomatix, Android-based application) [20], can also be used to extract the color intensities from the digital images. The result showed a high correlation between the concentration of glucose and the color change, described as color intensities of red, green and blue (RGB). The three-color RGB intensi-

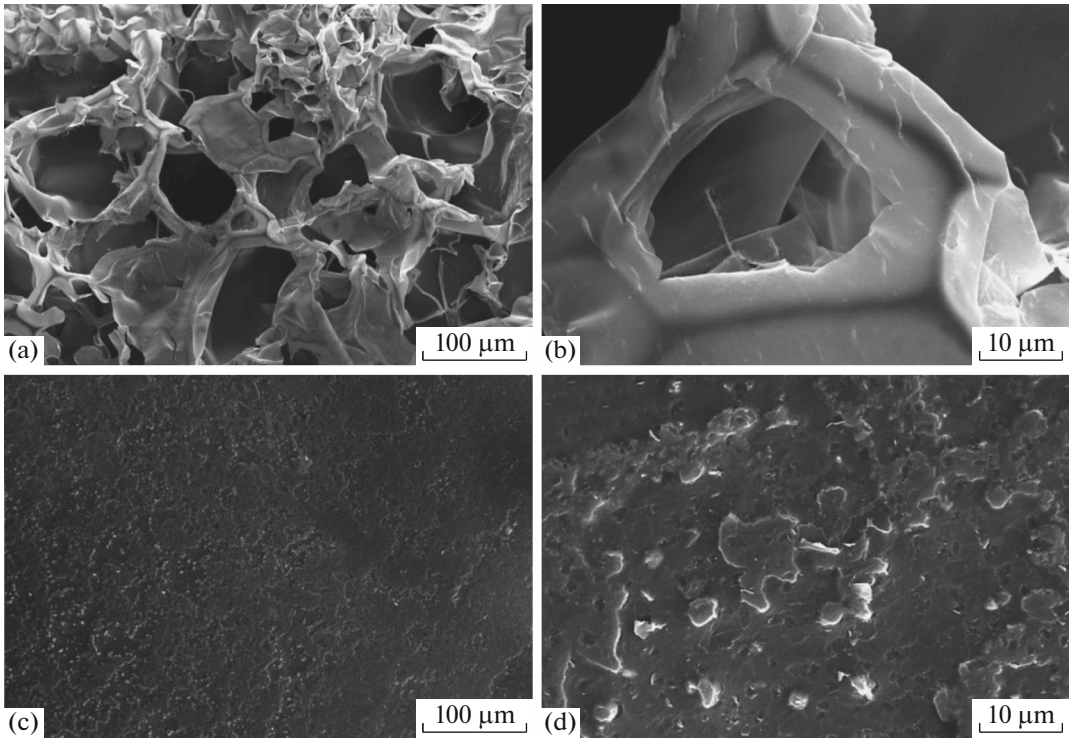

Fig. 2. SEM images of chitosan cryogel surface at ×250 (a) and ×2000 (b) showed a porous structure. The SEM image of non-cryogel chitosan showed a flat surface at ×250 (c) and rough surface without pores at ×2000 (d).

ties showed a high correlation, with the highest sensitivity (slope) was the decrease of the blue color intensity. Both red and green intensities increased with the increase of the glucose concentration with a lower sensitivity compared to the blue intensity. Therefore, for further study we used the blue color intensity.

Furthermore, the colorimetric detection using scanner was also playing an important role in reducing the analysis cost, since spectrophotometers or colorimeters are much more expensive. This detection method was also easy to replace using daily devices with their ability to record digital imaging, such as mobile phone, pocket or DSLR cameras.

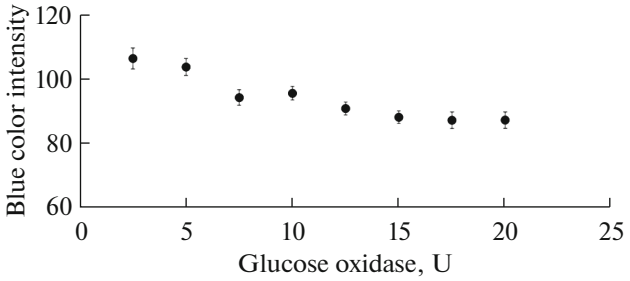

Fig. 3. Enzyme amount effect on the glucose biosensor response. Low blue color intensity provided a better response.

Enzymatic optimization. The amount of immobilized enzyme in the chitosan cryogel and the enzymatic reaction time were also studied. The use of a greater quantity of enzyme resulted in a better biosensor response However, the amount of enzyme was limited by the surface area of the chitosan cryogel, which was used as a supporting material for enzyme immobilization. Therefore, an excess of enzyme over the supporting material capacity would not increase the biosensor response. 50 μL of enzyme solution was used in this study with the concentrations of glucose oxidase enzyme between 2.5 and 20 units. The result showed that glucose biosensor responses increase with the increasing of glucose oxidase amount from 2.5 to 15 units. However, an additional amount of enzyme (15–20 units) resulted in a relatively similar glucose biosensor responses (Fig. 3). Thus 15 units of glucose oxidase were used for further fabrication.

Besides enzyme amount, it was also important to study the enzymatic reaction time. Longer contact between enzyme and substrate allowed to complete the reaction, resulting in better glucose biosensor responses. However, a longer reaction also increased the analysis time. The result showed the increase in biosensor responses from 1 to 5 min of the reaction time (Fig. 4) and relative stability after that. Thus, the reaction time of 5 min was used for further study.

Stability study. Chitosan cryogel showed superior stability during the biosensor development, which has

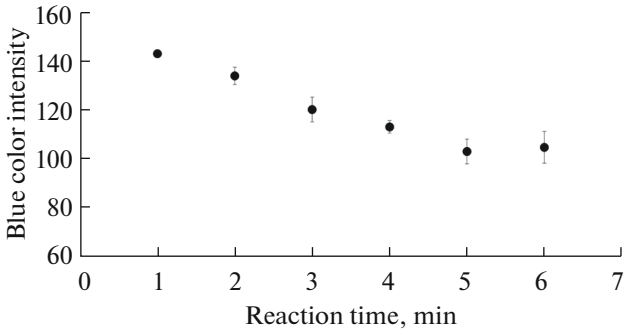

Fig. 4. Enzymatic reaction time effect on the glucose biosensor response. Lower blue color intensity represented a better visual response of the yellow color formed.

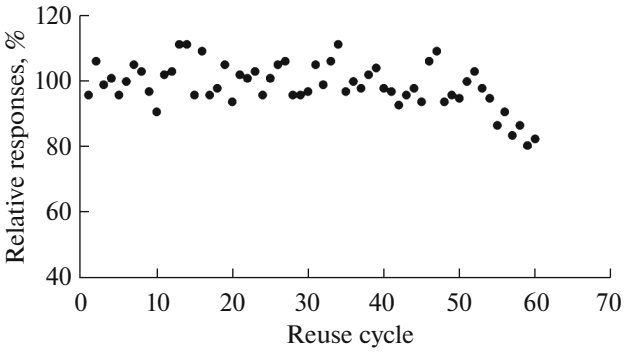

Fig. 5. Glucose biosensor operational stability performed by continuous determination of 3.0 mM glucose.

been reported previously for the amperometric glucose [1], the sialic acid [4] and microalbumin biosensors [5]. The fabricated glucose biosensor showed high stability of the immobilized glucose oxidase during the continuous analysis of 3.0 mM glucose solution for up to 56 measurements with the RSD of 5.47% (Fig. 5). The fabricated biosensor stability was much better than that of previously described based on alginate and stable for 10 measurements [21]. This can be possibly observed due to the improved enzyme activity maintenance by chitosan cryogel compared to the alginate cryogel. The ability of chitosan to improve the enzyme activity was presented in another study of urease immobilization, where the use of chitosan led to the higher activity than the application of alginate during reusability study [22]. The higher stability can arise from the stronger attachment of the hollow chitosan cryogel to the micropipette tip wall, whereas the chitosan cryogel beads were in a free physical contact with each other during the application of the suck-hold-release procedure for the analyte solution. This physical contact can influence the losses of the immobilized enzyme on the bead surface resulted in the remaining of the lower activity during reusability. The excellent reusability of the fabricated glucose biosensor can also lead to the much lower cost compared to the commercial glucose biosensor with disposable glucose oxidase strips.

Linear range. Other analytical characteristics of the fabricated glucose biosensor were also studied including linear range, limit of detection and limit of quantification. The fabricated glucose biosensor showed a linear response in the glucose detection from 0.5 to 3.0 mM with the regression equation $y = 38.31x - 3.08$ and $R^2 = 0.992$. The plotted data were the color intensity change of a series of glucose additions compared to the blank solution. For example, the solutions of 0 and 1.0 mM glucose showed the blue color intensity of 140 and 105, respectively. Thus, the color intensity change was 35. Plotting this data showed that the color intensities change with the increase of glucose concentration. Limit of detection (**LOD**) is the lowest amount of an analyte in a sample which can be detected but not necessarily quantified as an exact value [23], whereas limit of quantification (**LOQ**) is the concentration or amount of analyte quantifiable with a variation coefficient not higher than 10% [24]. The calculated LOD and LOQ were 0.26 and 0.87 mM, respectively.

Selectivity study. Glucose detection based on the use of glucose oxidase enzyme could be interfered by redox interferences, such as ascorbic and uric acids [15]. In this study, various amounts of ascorbic and uric acids were added to the standard glucose solution of 3.0 mM. The result showed there were no significant changes in glucose biosensor responses in the presence of these acids (Fig. 6), even at high concentrations of 1000 μM, which were exceeding their highest levels found in the blood, which are 500 μM for uric acid and 250 μM for ascorbic acid. The high selectivity of this glucose biosensor was supported by the use of titanium(IV) oxysulfate specific to the detection of hydrogen peroxide [17].

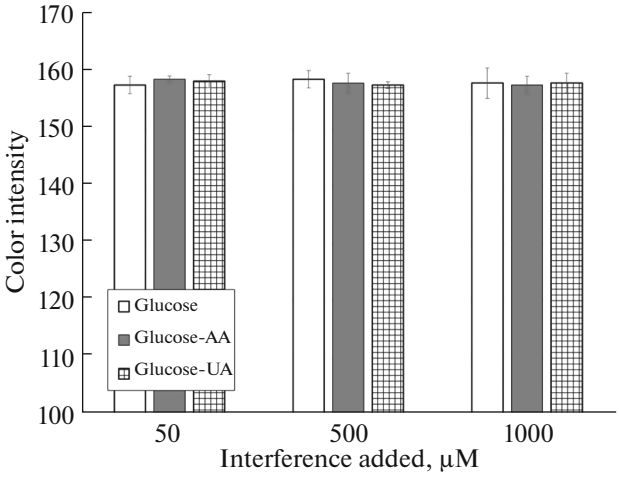

Fig. 6. Effect of ascorbic (AA) and uric (UA) acids on the glucose biosensor response.

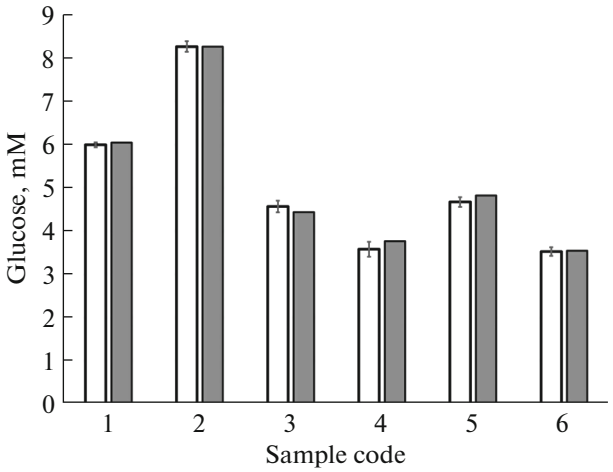

Fig. 7. Blood glucose determination using fabricated biosensor (white bar) compared to the spectrophotometric method used in hospitals (grey bar).

Blood glucose analysis. The applicability of the fabricated glucose biosensor was also studied using blood samples for glucose detection. Six samples of blood plasma were collected from a local hospital. The samples have been diluted three times with phosphate buffer solution (50 mM, pH 7) before analysis. The glucose detection of the blood plasma samples by the fabricated biosensor showed similar results (Fig. 7) to the standard spectrophotometric method performed in hospitals. Furthermore, the Wilcoxon Signed-Rank test was used to compare the results of the glucose determination by these two methods. Statistical analysis indicated that the results obtained by the fabricated biosensor compared to the standard method used in hospitals were not significantly different ($P = 0.225$, $P > 0.05$).

CONCLUSIONS

The colorimetric glucose biosensor was easy to prepare by immobilizing GOD in the chitosan cryogel in the micropipette tip. A relatively cheap instrumentation, consisting of a micropipette and commercial scanner, is required for the use of the proposed biosensor, thus it could be used in the low-income areas. Furthermore, the use of chitosan cryogel resulted in a high stability biosensor with a single preparation can be used for up to 56 measurements, which also reduced the cost. The analytical performance of the fabricated biosensor showed a good linear range, low limit of detection and high selectivity to common interferences found in the blood glucose determination, ascorbic and uric acids. Furthermore, blood glucose determination using the proposed biosensor compared to the standard method used in hospitals did not show a significantly difference in results (the Wilcoxon signed-rank test, $P > 0.05$).

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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