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Simple Colorimetric Glucose Biosensor using Chitosan Cryogel Supporting Material

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Abstract. Large surface area of porous structure cryogel showed an excellent matrix for enzyme immobilization, especially in the biosensor development. A chitosan cryogel beads was prepare by cross-linking chitosan with sodium tripolyphosphate at subzero temperature. This chitosan cryogel beads was then used to immobilize glucose oxidase for glucose biosensor fabrication. The biosensor was simple design and operation, using a micropipette tip to hold the immobilized enzyme, where the reaction can be performed by suck-hold-release the analyte using micropipette. The detection was based on the reaction between hydrogen peroxide, enzymatic product, with titanium (IV) oxysulfate to produce color change, which finally recorded using commercial scanner. The digital image obtained was then analyze using freeware of ImageJ to get the relationship between color change and the analyte concentration. The result showed a linear response in the glucose detection of 1.0 to 5.0 and 10 mM, with a regression of y = 11.33x + 46.02 and R2 of 0.983. The great enzyme immobilization was showed in the fabricated biosensor with 12 times uninterrupted analysis without reducing significant responses.

INTRODUCTION

Lifestyle changing including unhealthy diet, tobacco use and physical inactivity are the major risk factor of chronic disease of lifestyle (CDL). The main diseases of CDL leading causes of death in low and middle-income country such as heart disease, stroke, cancer, chronic respiratory and diabetes [1]. Diabetes also may occur during pregnancy which well known as gestational diabetes mellitus [2]. Diabetes complication causes several serious problems, such as premature morbidity, blindness, renal failure and amputation, while those problems may be reduced by early diagnostic [3]. Several analytical devices have been reported in the medical application such as chromatography [4], electrophoresis [5], spectrophotometry and biosensor. Biosensor is an analytical device consists of biological sensing element and a transducer which convert the biological recognition into measureable output signal [6]. Biosensor showed several advantages such as high selectivity, low-cost, high sensitivity, miniaturization ability and real-time capability, which these stimulates researchers to develop this method continuously. Biosensor performances can be improved their sensitivity and stability using cryogel, porous materials, for supporting material in the biosensor development [7,8].

Chitosan cryogel have been reported to improve biosensors performances of their sensitivity and stability in the biosensor development of glucose biosensor [9], sialic acid biosensor [10] and microalbumin biosensor [11]. The

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use of electrochemical detection in such previous studies have some limitation such as complex electrode preparation and relatively use an expensive instrument. Low-cost detection system have been reported for example cheap potentiostat [12], pocket camera, mobile phone camera [13] and commercial document scanner [14]. The use of color change detection showed several advantages such as simple instrument, easy to prepare standard solution and the color change some time can be easily detected by naked eyes [15].

In this work, the chitosan cryogel was used to immobilize glucose oxidase enzyme in the glucose biosensor fabrication. The biosensor design would simple operation using immobilized enzyme in micropipette tips, operating by sucking and pumping the sample solution. The color change was recorded using commercial document scanner for easy operation and cheap detection system.

MATERIALS AND METHODS

Materials

Chitosan from crab shell, Glucose oxidase (GOD) (EC 1.1.3.4, Type II-S, 15-50 unit mg^{-1}) and Titanium(IV) oxysulfate were from Sigma-Aldrich (Steinheim, Germany). D-(+)-glucose anhydrous (≥ 98.0 %),penta-Sodium triphosphate, hydrogen peroxide, acetic acid, sodium dihydrogen o-phosphate and disodium hydrogen o-phosphate were from Merck (Germany).

Apparatus and measurements

Scanning electron microscopy (SEM) Table Top TM3000 (Hitachi, Japan) was used to characterize the morphology of the chitosan cryogel. The color change was measured using a Shimadzu Biospec 1601 UV-Vis spectrophotometer (Shimadzu, Japan) and commercial document scanner (Canon LiDE 120, Canon Inc, Vietnam). The chitosan cryogel biosensor preparation and measurement were used a 10-100 µL micropipette (Eppendorf, Germany).

Chitosan cryogel-GOD Preparation

The chitosan cryogel beads was prepare using chitosan solution of 2% (w/v) in a 1% (v/v) of acetic acid solution. The chitosan solution of 100 µL was dropped to a TPP solution of 1% (w/v) to prepare the chitosan beads. The polymerization was performed at room temperature for 30 min, continued by freezing at -20 ^{0}C for 6 h to allow cryogelation process. The chitosan cryogel beads were then thawed at 4 ^{0}C for 1 h and activated using TPP solution for enzyme immobilization. Glucose oxidase (GOD) enzyme of 1 U/uL^{-1} was prepared in a 50 mM of phosphate buffer pH of 7.0. Chitosan cryogel beads were then soaked in the 50 µL of GOD solution and kept for 12 h to complete the cross-linking between GOD and chitosan cryogel beads. The chitosan cryogel-GOD beads were placed in a 100 µL micropipette tip and ready to use for glucose biosensor measurement. The brief of the biosensor fabrication was described in the Figure 1.

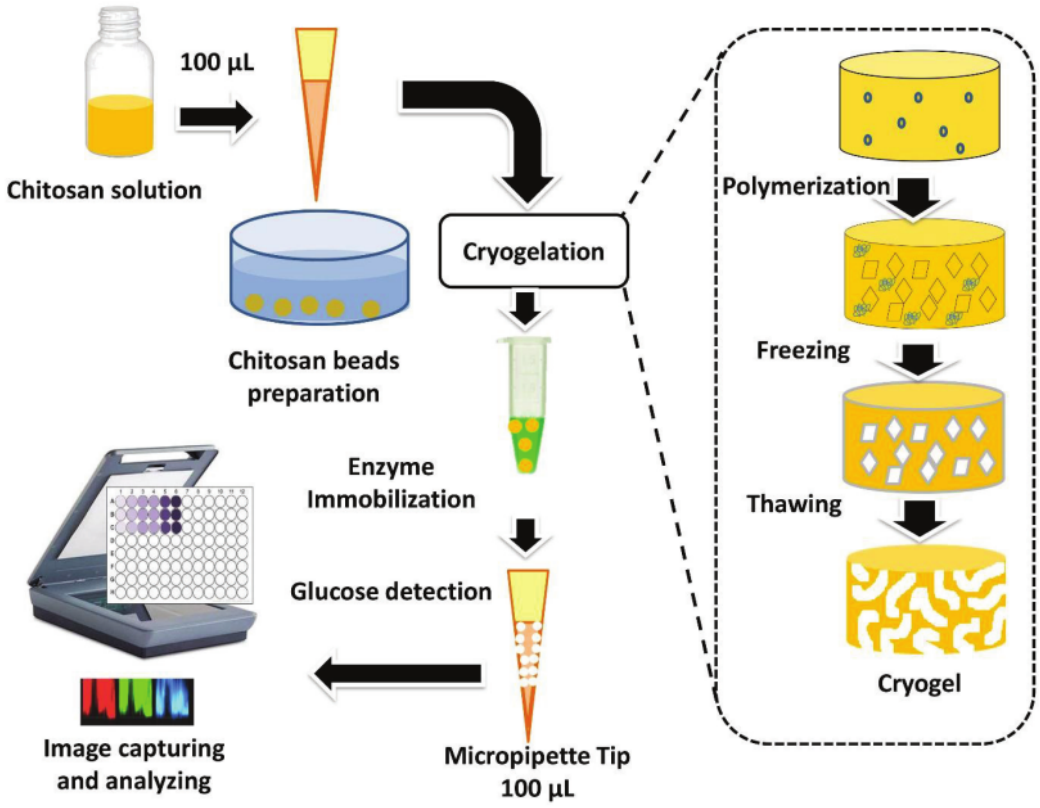

FIGURE 1. Chitosan-GOD cryogel for glucose biosensor preparation

Glucose biosensor detection

The glucose biosensor detection was based the reaction of glucose and glucose oxidase enzyme, resulted in hydrogen peroxide, which can be detected using specific indicator. The hydrogen peroxide detection was based on procedure previously reported [16] with few modification. An indicator solution was prepared by adding 2.5 g of Titanium (IV) oxysulfate to a 100 mL of 2 M sulfuric acid solution. Reaction was performed in a flat bottom 96-well plate by mixing 50 µL of titanium solution and 100 µL of sample. The changes of indicator color in the hydrogen peroxide detection was first studied using standard solution of 1.0 to 5.0 mM hydrogen peroxide. The glucose measurement was then performed by filling the in-tip chitosan-GOD cryogel with 100 µL of glucose sample, allowed the enzymatic reaction for 5 minutes and the reaction product was dropped to the 50 µL titanium solution in the 96-well plate. This simple biosensor was operating by micropipette sucking, holding and pumping. The color change by hydrogen peroxide or enzymatic glucose product was recorded using a scanner. The ImageJ Software was used to analyze the color change images due to hydrogen peroxide concentration. The relation between glucose concentrations and color changes were then plotted in the regression line to get the linear equation of $y = mx + b$.

Stability study

The stability of the fabricated glucose biosensor was the ability of immobilized GOD enzyme in the cryogel matrix to allow several reactions without significantly lost its activity. The study was performed by uninterrupted

measuring glucose solution of 3 mM, using the glucose detection procedure previously describe. The operational stability was determined by the biosensor response given for more than 90% in a series measurement.

RESULTS AND DISCUSSION

Chitosan cryogel

One strategy to improve biosensor performance is sensitivity enhancement. For the enzyme-based biosensor, the sensitivity can be improved by the use of a large surface area supporting material for enzyme immobilization. Porous material such as cryogel has large surface area, thus widely used as supporting material in the biosensor development [7, 9, 10]. Cryogel can be prepared using several polymers such as polyvinyl alcohol, chitosan and alginate. This study use chitosan to prepare the cryogel beads crosslinked by TPP. In this condition, amine groups ($-NH_3^+$) of chitosan or enzyme were easily crosslinked by poly-anion solution to make the chitosan beads. The advantages of this method were simple preparation and using aqueous solution which relatively less toxic than organic solvent. The cryogelation allowed water as solvent to freeze, resulted in porous structure during thawing (Fig. 2). In the cryogelation process, the chitosan and crosslinker remain in the semifrozen or unfrozen phases and form a cross-linked network, while the ice crystals nucleated from the aqueous phase during freezing, act as porogens, resulted in porous structure during thawing (Figure 2). The chitosan cryogel beads was of about 2-3 mM, with pores size diameter of 50-100 μm.

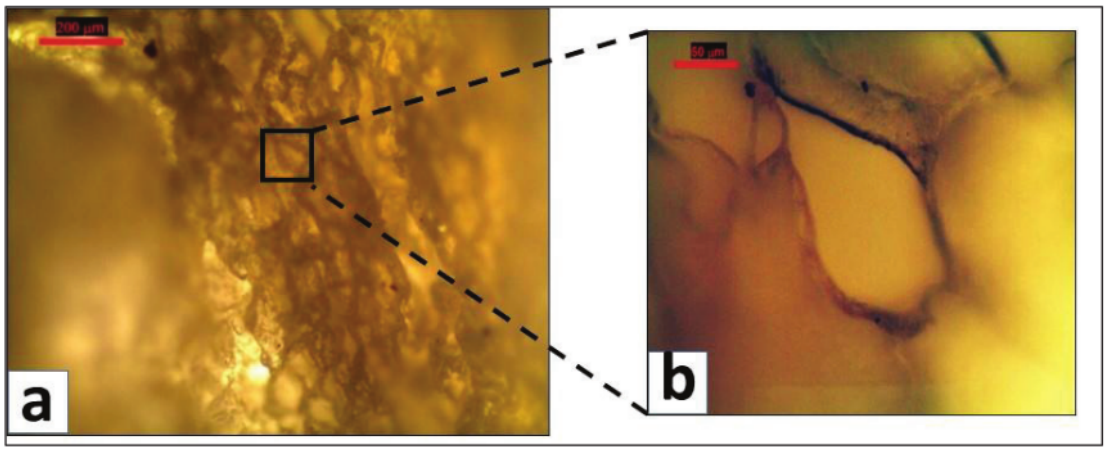

FIGURE 2. Chitosan cryogel bead surface showed a porous structure using binocular microscope at x100 (a) and x400 (b).

Glucose biosensor detection

The fabricated glucose biosensor detection was based on enzymatic reaction of glucose with glucose oxidase resulted in hydrogen peroxide according to the following reaction below (1).

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

$$Ti^{4+} + H_2O_2 + 2\,H_2O \longrightarrow H_2TiO_4\ _{(pertitanic\ acid\ =\ yellow)} + 4\,H^+ \quad (2)$$

The glucose concentration would equal to the hydrogen peroxide produced, which further colorimetric detected using titanium(IV) oxysulfate according to the reaction above (2). The color change with the increasing glucose concentration was studied in the range of 1.0 to 5.0 and 10 mM. The resulted series color change was then recorded using commercial scanner (Fig. 3b), following by analyzing their color intensity change (red, green or blue) using free software of ImageJ (imagej.nih.gov/ij/) to get RGB profile (Fig.3b). The relation between color intensity change and glucose concentration was selected from the three color of red, green or blue with highest sensitivity and best regression coefficient. The result showed the reducing blue color intensity was the best one (Fig. 3a).

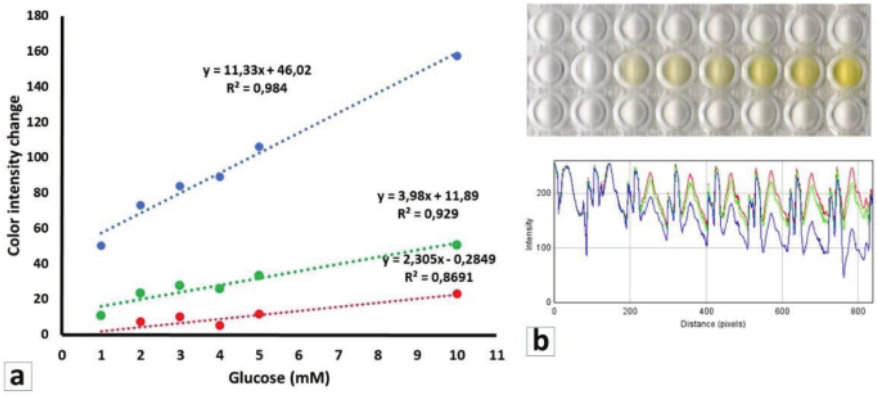

FIGURE 3. (a) Calibration curve of fabricated chitosan-GOD cryogel beads glucose biosensor to detect a series glucose concentration (1.0 – 5.0 and 10 mM). (b) the example color change with the glucose concentration in a 96-well plate recorded by scanner and the color intensity profile analyzed by ImageJ software.

Operational stability study

Previous study showed a high stability of biosensor based on cryogel as enzyme immobilization supporting material, due to the cryogel allowed to hold the enzyme activity [9, 10]. However, various polymer as cryogel backbone and various matrix form may resulted in the different enzyme immobilization stability, due to their characteristic, for example, natural polymer such as chitosan usually more biological activity friendly than synthetic polymer such as polyvinyl alcohol. The operational stability of the fabricated glucose biosensor was showed a good operational stability with similar responses up to 12 uninterrupted analyses (Figure 4). This high glucose biosensor may due to the chitosan cryogel structure a combination of chemical and mechanical stability and is ideal for the immobilization of enzymes and cells [9].

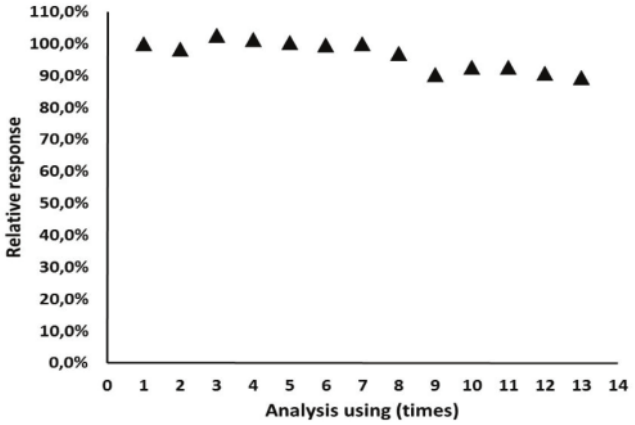

FIGURE 4. Chitosan-GOD cryogel glucose biosensor stability study showed relative stable responses up to 12 analysis repetitions of of 3.0 mM glucose solution.

CONCLUSION

The glucose biosensor was fabricated based on chitosan cryogel as GOD immobilization supporting material and simple colorimetric method of hydrogen peroxide detection recorded by commercial scanner. The chitosan-GOD cryogel biosensor showed a good linearity in the glucose detection of 1 to 10 mM. Furthermore, the biosensor showed a good enzyme immobilization with it showed good responses up to 12 times uninterrupted analysis without a significant responses decreasing. This simple and good performance biosensor would be great to apply in other applications, especially enzyme based biosensor.

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