Gmail **Amin Fatoni <aminfatoni@gmail.com>**

Journal of Analytical Chemistry

1 message

Журнал аналитической химии <zhakh@geokhi.ru> Thu, Mar 2, 2017 at 3:02 PM
Reply-To: zhakh@geokhi.ru
To: aminfatoni@gmail.com

Dear authors,
We received your new manuscript
The registration number is 68

Thank you

Best regards,
Editor Filatova-- Journal of Analytical Chemistry

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Amin Fatoni <aminfatoni@gmail.com>

Journal of Analytical Chemistry

2 messages

Журнал аналитической химии <zhakh@geokhi.ru> Tue, May 16, 2017 at 2:20 PM
Reply-To: zhakh@geokhi.ru
To: aminfatoni@gmail.com

Dear authors,

1.We are sending you the manuscript No 68 for the revision . We ask you to introduce the necessary amendments into the text of your manuscript , taking into considerations the remarks of the reviewer and reviewer and Guidelines of the Journal of Analytical Chemistry.

2. 2. Please include paper titles in the references list. This should be done for all journal papers and collections of articles (see Authors' Guidelines at the Journal site).

Sincerely yours,

Editor Filatova, Journal of Analytical Chemistry

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Amin Fatoni <aminfatoni@gmail.com> Tue, May 16, 2017 at 7:39 PM
To: zhakh@geokhi.ru

Dear Filatova,
Editor Journal of Analytical Chemistry

Thank you for the review result. I will revise accordingly and send back as soon as possible.

Regards,
Amin Fatoni
[Quoted text hidden]
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Amin Fatoni

Gmail **Amin Fatoni <aminfatoni@gmail.com>**

Manuscript No 68 Revision

2 messages

Amin Fatoni <aminfatoni@gmail.com> Tue, May 23, 2017 at 9:51 PM
To: zhakh@geokhi.ru

Dear Filatova,
Editor Journal of Analytical Chemistry

I have revised the manuscript according to the Editor and Reviewer suggestion.
The following files are attached:
1. Replayed Editor and Reviewer comments (N 68 Amin Fatoni - Replayed)
2. Revised manuscript (Fatoni_manuscript_Revision)

Best regards,

--
Amin Fatoni

2 attachments

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Журнал аналитической химии <zhakh@geokhi.ru> Wed, May 24, 2017 at 6:41 PM
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To: Amin Fatoni <aminfatoni@gmail.com>

Dear authors,
This is confirm that we received your manuscript № 168 after correction

Thank you

Best regards,
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Journal of Analytical Chemistry

4 messages

Журнал аналитической химии <zhakh@geokhi.ru> Wed, Feb 13, 2019 at 8:30 PM
Reply-To: zhakh@geokhi.ru
To: aminfatoni@gmail.com
Cc: aminfatoni@gmail.com

Dear Authors,

Your manuscript № 68(17) has been edited. We ask you to check the changes done by the editor and reply to the questions (if any). All changes and questions are shown as notes, green colored text and text highlighted in color. The Track Changes option of MS Word must be "on". If you agree with the changes, please "accept" them. If you disagree, please create a comment explaining why you cannot accept the change.

When updating you manuscript, please, be sure to work with this (edited) version of the file. Do not use your older versions.

It is also very important to correct the Figures according to editor's comments (see attached pdf file).

We ask you to give a serious consideration to this work, as this is the last step when you can make changes in the manuscript. At the step of the galley proofs, no corrections against the original will be accepted.

We ask you to send the manuscript as MS Word file as soon as possible to zhakh@geokhi.ru. This should be one MS-Word file containing the sections in the following order.

1. **Text**
2. **Captions for Figures**
3. **References**
4. **Tables**
5. **Figures (all the figures should be in black-and-white).**

On the title page of the Copyright Transfer Agreement indicate the names and surnames of all authors

With best regards,

Editorial Office of the Journal of Analytical Chemistry

3 attachments

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Amin Fatoni <aminfatoni@gmail.com> Thu, Feb 14, 2019 at 7:38 AM
To: zhakh@geokhi.ru

Dear Editor of the **Journal of Analytical Chemistry**,

Thank you for the article proof.
I will revise accordingly and return to you as soon as possible.

Best regards,
Amin Fatoni
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Amin Fatoni

Amin Fatoni <aminfatoni@gmail.com> Fri, Feb 15, 2019 at 8:49 AM
To: zhakh@geokhi.ru

Dear Editor of **the Journal of Analytical Chemistry**,

I have checked the article, revise accordingly, fix the references and figure (attached).
I have also filled and sign the copyright form.

Please contact me if any other information is needed.

Best regards,
Amin Fatoni

On Wed, Feb 13, 2019 at 8:30 PM Журнал аналитической химии <zhakh@geokhi.ru> wrote:
[Quoted text hidden]

--
Amin Fatoni

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2 attachments

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Журнал аналитической химии <zhakh@geokhi.ru> Fri, Feb 15, 2019 at 3:45 PM
Reply-To: zhakh@geokhi.ru
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Dear authors,

We received the final version of manuscript № 68(17(and a Copyright Transfer

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Gmail Amin Fatoni <aminfatoni@gmail.com>

Proof_AnChem9_19(21.07.19)

2 messages

ced <cedgraph@pleiadesonline.com> Thu, Jul 18, 2019 at 2:53 PM
To: aminfatoni@gmail.com

Dear Author,

Please find attached the proofs of your article which will be published in
Journal of Analytical Chemistry № 9_19.

PLEASE read the instructions attached to this e-mail message (Instr_Rus.pdf in Russian or Instr_Eng.pdf in English).
ATTENTION: OUR INSTRUCTIONS ARE PERIODICALLY UPDATED. The instructions contain guidelines and recommendations, without the compliance of which your reply letter will be lost during automatic processing.

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Sincerely,
****
LLC «PBC «AKADEMKNIGA»

Уважаемый Автор,

Направляем Вам для ознакомления корректуру Вашей статьи, которая готовится к публикации в журнале
Journal of Analytical Chemistry № 9_19.

УБЕДИТЕЛЬНО ПРОСИМ Вас после получения данного письма прочитать инструкцию в приложении (файл Instr_Rus.pdf с инструкцией на русском языке или файл Instr_Eng.pdf с инструкцией на английском языке). ОБРАЩАЕМ ВНИМАНИЕ: НАШИ ИНСТРУКЦИИ ПЕРИОДИЧЕСКИ ОБНОВЛЯЮТСЯ. Инструкция содержит правила оформления и рекомендации, без выполнения которых Ваше сообщение будет потеряно при автоматической обработке ответов.

ОБРАТИТЕ ВНИМАНИЕ, что после получения Вашего ответа (или его отсутствия по истечении указанного ниже срока) оригинал-макет Вашей статьи будет отправлен в типографию без дальнейших стадий проверки и согласования.

Исправления и замечания должны быть внесены непосредственно в PDF-файл статьи. PDF-файл с исправлениями просим высылать ответом на данное письмо не позднее 21.07.19. В ответном письме должен быть либо файл с исправлениями, либо сообщение в тексте письма об отсутствии замечаний.

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С уважением,
****
ООО «ИКЦ «АКАДЕМКНИГА»

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Amin Fatoni <aminfatoni@gmail.com> Sat, Jul 20, 2019 at 7:25 AM
To: ced <cedgraph@pleiadesonline.com>

Dear Journal of Analytical Chemistry Editor,

Thank you for your email.
I have two comment on the article (commented PDF file attached) as follows:

1. Suggestion to put the **Conclusions** session after the **Fig. 7**
2. Remove the unused word in the end of the article.

Best regards,
Amin Fatoni

[Quoted text hidden]
--
Amin Fatoni

AnChem1909002AminFatoniKOR-proofed.pdf
2138K

We would like to thank the reviewers and the editor for the suggestions and comments. The manuscript has been revised accordingly and the responses to the queries are as follows.

Editor Comment:

1.We are sending you the manuscript No 68 for the revision . We ask you to introduce the necessary amendments into the text of your manuscript , taking into considerations the remarks of the reviewer and reviewer and Guidelines of the Journal of Analytical Chemistry.

Reviewer question and suggestion have been applied to revise the manuscript.

2. 2. Please include paper titles in the references list. This should be done for all journal papers and collections of articles (see Authors' Guidelines at the Journal site).

All references have been added their title

Review Comment:

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

Amin Fatoni et. al.

The article corresponds to the profile of the Journal of Analytical Chemistry and can be published after corrections in accordance with the following remarks.

1. The first subsection of the section "Results and their discussion" should be moved to the "Experimental part".

I have move the first subsection of "Results and their discussion" to the "Experimental part", the correction was mark in green text.

2. It is necessary to justify the choice of titanium (IV) oxysulfate as reagent. The matter is that the compounds of titanium (IV) in aqueous solutions undergo hydrolysis and pass into polymeric forms of complex composition and structure.

The titanium sulfate method for H_2O_2 detection was selected due to their instantaneous reaction, forming yellow-colored complex which is stable for at least 6 hours [1]. The method is specific to H_2O_2 and there are no interferences from other compounds present in water [2]. The reaction working under strong acidic conditions would make it independent to the pH[1].

This reasons have been added to the discussion, mark in green text.

3. In scheme (1) on page 7, the non-existing Ti4 + cation should be replaced by the real Ti (IV) form present in the solution under these conditions. The hydrated Ti4 + ion can not exist due to a large charge in solutions.

Thank you for the correction, since Ti4 + would not occur in ion form of aqueous solution, we change the scheme with a Ti – ligand complex form of Ti(IV)OSO4 (Titanium(IV)oxysulfate.

4. It is useful in the "Introduction" section to formulate the research objective.

The research objective has been added in the "Introduction", the additional was mark in green.

5. The authors characterize the developed sensor as a "diagnostic and accurate instrument" (p.3). However, the term accuracy typically refers to a technique for determining by an instrument, rather than to an analyte detection device.

Thank you for the suggestion. We have change the term "diagnostic and accurate tool" to "diagnostic and promising detection device". The change was marked in green text.

6. Reference should be made to the literature source, in which the "Wilcoxon signed-rank" methodology is described.

Wilcoxon signed-rank is a nonparametric test procedure for the analysis of matched-pair data [3]. It can be used for comparing two related sample (new method vs standard methods for example) as an alternative to the paired Student's t-test when the population cannot be assumed to be normally distributed [4] (only few sample tested, much lower than 30 samples).
Reference has been added to the methodology section.

7. References to literature in the text should be enclosed in square brackets.

The references have been revise according to the suggestion

8. It is necessary to explain what the authors mean by the LOQ and LOD. Reference to Fig. 7 is not enough for this.

The explanation of LOQ and LOD have been added, mark in green text.

9. In many places in the text it is necessary to edit the English language.

Some corrections have been made, mark in blue font color.

References of this explanation

[1] Z. Machala, B. Tarabova, K. Hensel, E. Spetlikova, L. Sikurova, P. Lukes, Formation of ROS and RNS in Water Electro-Sprayed through Transient Spark Discharge in Air and their Bactericidal Effects. *Plasma Processes Polym.* 10(2013) 649-59.
[2] C.N. Satterfield, A.H. Bonnell, Interferences in titanium sulfate method for hydrogen peroxide. *Anal Chem.* 27(1955) 1174-5.
[3] R.F. Woolson, Wiley Encyclopedia of Clinical Trials, John Wiley & Sons, Inc.2007.
[4] R. Lowry, Concepts and applications of inferential statistics. (2014).

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

Amin Fatoni

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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, which then a titanium (IV) oxysulfate was used to measure the hydrogen peroxide, indicated by yellow color formed. The color change with the concentration then recorded by a commercial scanner and analyzed using ImageJ software. The fabricated biosensor allows easy to prepare by in-tips enzyme immobilization and user-friendly operation using micropipette by the suck-hold-release method to detect glucose. The enzyme immobilization has been optimized including enzyme amount and reaction time. The biosensor showed a high operational stability up to 56 analysis in a single immobilized enzyme, with a wide linearity (0.3 to 3.0 mM glucose), high specificity, and also agreed with the standard method used in the hospital to detect blood glucose (Wilcoxon signed-rank, $P > 0.05$).

Keywords: cryogel, glucose biosensor, chitosan, colorimetric.

INTRODUCTION

Early detection and diagnostic require less treatment and are less cost of diseases management. Glucose detection in the blood is the most feasible approach for early detection of diabetes mellitus. Several methods to detect blood glucose level have been reported, including chemical, enzymatic and affinity-based detection principles. A biosensor that utilized biosensing element to detect glucose showed advantages of their high selectivity due to the nature of the biosensing, for example, enzyme glucose oxidase, catalyze a high specific substrate of glucose. Many strategies have been reported to improve the glucose biosensor performances, which mainly performed to increase the sensitivity, selectivity, and stability or to decrease the analysis time analysis cost.

High stability biosensor could be achieved by maintenance the enzyme activity by the use of natural polymers as a supporting material such as chitosan [1], agarose [2] and alginate [3]. Chitosan could be prepared in cryogel form, which provided a high surface area, high stability and also high sensitivity in the biosensor development to detect glucose [1], sialic acid [4], microalbumin [5], and carcinoembryonic antigen [6]. However, the use of electrochemical detection in the previous chitosan cryogel based biosensors showed some disadvantages such as a high-cost instrument, a laborious electrode preparation, and a relative complex operation.

On the other hand, colorimetric based sensors have received considerable attention due to their simplicity, high sensitivity, and low cost. The rapid growth of digital imaging device, proposed cost-effective methods in the analysis based on the color change with the analyte concentration, for example, the use of smartphone camera [7], web-cam [8], digital camera [9] and scanner [10]. The ability of such devices to take digital images have been reported as function as a spectrophotometer with a wavelength resolution of 5 nm [11] which wide range application such as flow immunoassay [12], quantum-dot labeling bacteria [13] and label-free

photonic crystal biosensor [14].

The aimed of this work was to develop simple preparation and low-cost colorimetric glucose biosensor using chitosan cryogel as 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 measured by a commercial document scanner. The biosensor was also easy to operate by a suck-hold-release method using a micropipette, similar to daily used of the micropipette in the liquid sample handling. This biosensor would be a user-friendly diagnostic and promising detection device, compared to the standard methods to detect glucose.

EXPERIMENTAL

Chemicals and Materials

Glucose oxidase (GOD) (EC 1.1.3.4, Type II-S, 15-50 unit mg-1), 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 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 the digital image of samples color changes. An ImageJ software (https://imagej.nih.gov/ij/) was used to analyze the digital imaging into RGB (red, green, blue) intensity. Flat bottom 96 well plate (Iwaki, Japan) was used as sample holder for reaction and measurement.

Biosensing Element Preparation

The biosensing element was based on the immobilized glucose oxidase in the chitosan cryogel (Figure 1). Chitosan solution was prepared by dissolving 2 g in 80 ml of 1% (v/v) of acetic acid, stirred gently with heating and adjust with 1% acetic acid solution to make 100 ml of solution. The chitosan solution was then filtered and kept at 4°C when not in use. Chitosan cryogel has been prepared by simultaneously polymerization and freezing of chitosan solution using glutaraldehyde as a crosslinker. Chitosan solution of 100 µL was mixed with five µL glutaraldehyde solution of 2.5%. This mixture was then added to a micropipette tip of 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 h to allow the cryogelation process. Chitosan polymers have been crosslinked, while water solvent was frozen allowed porous cryogel backbone build. The chitosan cryogel was then thawed at 4 °C to allow crystal water melt, continued with removing the stainless steel rod to produce the hollow chitosan cryogel in the micropipette tips (hChiCryo). The developed hChiCryo inside a blue micropipette tip (100-1000 µL) was easy to prepare, which this tip had a double function, as a shield of the hChiCryo from physically damage and also 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 enzyme was prepared in the phosphate buffer solution (50 mM, pH of 7.0) with the enzyme concentration of 0.2 U/µL. This enzyme solution of 50 µL was immobilized in the activated hollow chitosan cryogel for six h at 4 °C. This hChiCryo-GOD was kept in 4°C when it was not in use.

Fig. 1 here

Glucose Detection

Glucose detection in the fabricated biosensor was based on the enzymatic reaction of

glucose and GOD enzyme. The GOD convert glucose into gluconolactone and hydrogen peroxide. Series concentration (0.6 – 1.4 mM) of glucose solutions of 100 µL 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. Titanium(IV)oxy sulfate solution (2.5% b/v in 2 M of sulfuric acid) of 50 µL was then added to each solution. The well plate was then placed in the document scanner, scanned and analyzed the RGB intensity using ImageJ software. The RGB intensity related to the hydrogen peroxide concentration was analyzed. As a comparison, the color change of the hydrogen peroxide and indicator were also analyzed using a spectrophotometer.

Enzymatic Optimization

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

Stability Study

One of the advantages in the use of an immobilized enzyme, such in this fabricated biosensor, is the reusability. The reusability of the biosensor in the glucose detection was studied by measuring 3.0 mM standard glucose solutions uninterrupted. 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 the blood sample are ascorbic acid and uric

acid [15]. These interferences could result in positive false in some glucose detection based on the use of glucose oxidase, as a reducing agent, similar to the hydrogen peroxide resulted 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 the various concentrations of ascorbic acid and uric acid.

Real Sample Analysis

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

RESULTS AND DISCUSSION

Chitosan Cryogel Preparation and Morphological Study

SEM image of the fabricated chitosan cryogel showed pores diameters of 10-100 µm (Figure 2 a,b). The pores size of this chitosan cryogel were slightly larger than chitosan-albumin cryogel of 5-20 µm previously reported [1]. It has also been confirmed that the non-cryogel chitosan, polymerization at 4 °C, did not show a porous structure (Figure 2, c,d).

Fig. 2. here

Glucose Detection

This biosensor was designed as user-friendly operate called the suck-hold-release method. First, glucose solution was sucked using micropipette armed with hChiCryo-GOD tip, and then the glucose was held in the tip as enzyme reactor to allow glucose conversion. Finally, the resulted hydrogen peroxide was released from the micropipette tip, and it was reacted with the indicator according to the reaction below. Glucose was first converted to gluconic acid and hydrogen

peroxide by glucose oxidase enzyme immobilized in the chitosan cryogel (1). The resulted hydrogen peroxide was then reacted with titanium(IV) oxysulfate to produce a yellow color of pertitanic acid (2).

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

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

The use of titanium sulfate method for H_2O_2 detection was selected due to their instantaneous reaction and forming a yellow-colored complex which was stable for at least 6 hours [17]. The method was also highly specific to H_2O_2 detection without any interferences responses [18]. Furthermore, the strongly acidic solution would make it independent of the pH [17].

This mixture was prepared in the 96 well plate flat bottom to easy capture the color change using a scanner, with further analyzed using ImageJ software. Similar software was also could be used to extract the color intensity from the digital imaging such as Adobe Photoshop™ (Adobe Inc, Windows-based application)[19] and Color Grab (Loomatix, Android-based application) [20]. The result showed a high correlation between glucose concentration and color change, described as color intensity of red, green and blue (RGB) (Figure 3). The three color intensity (R, G, and B) showed a high correlation, with the highest sensitivity (slope) was the decreasing of blue color intensity. Both red and green intensities were increased with a lower sensitivity than that of blue intensity. Therefore, for further study would use the blue color intensity. Furthermore, the colorimetric detection using scanner was also playing an important role to reduce the analysis cost, by avoiding the high cost of spectrophotometer or colorimeter. This detection method was also easy to replace using daily devices with their ability to record digital imaging such as mobile phone camera, pocket camera or DSLR camera.

Fig. 3. here

Enzymatic Optimization

This study including the amount of enzyme immobilized in the chitosan cryogel and the enzymatic reaction time. More enzyme used would result in a better biosensor response due to the high enzymatic product. However, this condition should be limited by the surface area of the chitosan cryogel as supporting material in the enzyme immobilization. Therefore, excess enzyme over the supporting material capacity would not increase the biosensor responses. This study was used 50 μL of enzyme solution with a various concentration of 2.5 to 20 unit of glucose oxidase enzyme. The result showed the increasing glucose biosensor responses with the increasing of glucose oxidase amount of 2.5 to 15. However, an additional enzyme of 15 to 20 unit resulted in a relatively similar glucose biosensor responses (Figure 4). Thus the 15 unit of glucose oxidase was used for further fabrication.

Fig. 4. here

Fig. 5. here

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

Stability Study

Chitosan cryogel showed superior stability in the biosensor development, which has been reported previously in the amperometric glucose biosensor [1], the sialic acid biosensor [4] and microalbumin biosensor [5]. The fabricated glucose biosensor showed a high stability of the immobilized glucose oxidase with uninterrupted analysis of 3.0 mM glucose solution for up to 56 analysis with an RSD of 5,47% (Figure 6). The fabricated biosensor stability was much better than that of our previous work using alginate of 10 analysis[21]. The better stability may

due to the chitosan cryogel had better enzyme activity maintenance then the alginate cryogel. The use of chitosan to improve the enzyme activity was agreed with another study of urease immobilization, where using chitosan remains higher activity than alginate during reusability study[22]. The fabricated hollow chitosan cryogel was also much better than chitosan bead of 12 analysis[23]. The higher stability may due to the hollow chitosan cryogel was strongly attached to the micropipette tip wall, whereas the chitosan cryogel beads were freely physical contact each other during operational of suck-hold-release the analyte solution. This physical contact may effect to the losses of immobilized enzyme on the bead surface resulted in the lower activity remains during reusability. The excellent reusability of the fabricated glucose biosensor would also be much lower cost compared to the commercial glucose biosensor with disposable glucose oxidase strip.

Fig. 6. here

Linear Range

Other analytical performances of the fabricated glucose biosensor have also been 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 mM to 3.0 mM with regression equation of $y = 38{,}31x - 3{,}08$ and R^2 of 0.992. The plotted data were the color intensity change of 0 mM glucose compared to a series glucose concentrations additions. For example, the solution of 0 mM glucose concentration showed the blue color intensity of 140 and the solution of 1.0 mM glucose showed the blue color intensity of 105. Thus, the color intensity change was 35. By plotting this data, the color intensities change increased with the glucose concentrations. Limit of detection (LOD) is the lowest amount of an analyte in a sample which can be detected but not necessarily quantified an exact value [24], whereas limit of quantification (LOQ) is the concentration or amount of analyte quantifiable with a variation coefficient not higher than 10% [25]. The calculated LOD was 0.26 mM and calculated LOQ

was 0.87 mM (Figure 7).

Fig. 7. here

Selectivity Study

Glucose detection based on the use of glucose oxidase enzyme could interfere by redox interferences such as ascorbic acid and uric acid [15]. In this study, various ascorbic acid and uric acid have been added to standard glucose solution of 3.0 mM. The result showed there were no significant glucose biosensor responses change in the present of ascorbic acid and uric acid (Figure 8), even in the high concentration of 1000 µM which were higher than their highest level found in the blood for uric acid of 500 µM and ascorbic acid of 250 µM. The high selectivity of this glucose biosensor supported by the use of titanium (IV) oxysulfate which was specific to H_2O_2 detection [17].

Fig. 8. here

Fig. 9. here

Blood Glucose Analysis

The analytical performance of the fabricated glucose biosensor was also studied in the blood sample glucose detection. Six samples of blood plasma were collected from a local hospital. The samples have been diluted three times with phosphate buffer (50 mM, pH of 7) before analysis. The glucose detection of the blood plasma samples by the fabricated biosensor showed similar results (Figure 9) to the standard spectrophotometric method performed by the hospital. Furthermore, statistical analysis of Wilcoxon Signed Rank test was used to compare the glucose concentration of those two methods. Statistical analysis indicated that the glucose concentration obtained by the fabricated biosensor compare to the standard method of the hospital used were not significantly different ($P = 0.225$, $P>0.05$).

CONCLUSIONS

The colorimetric glucose biosensor has been easy to prepare by immobilizing GOD in the chitosan cryogel growth in the micropipette tip. This biosensor used a relative cheap instrumentation of micropipette and commercial scanner, which could be applied in the low-income area. Furthermore, the use of chitosan cryogel resulted in a high stability biosensor with a single preparation can be used up to 56 analysis, which would also reduce the analysis 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 analysis of ascorbic acid and uric acid. Furthermore, blood glucose analysis using the biosensor compare to the standard method used in the hospital did not show a significantly different (Wilcoxon signed-rank, $P > 0.05$)

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References

[1] A. Fatoni, A. Numnuam, P. Kanatharana, W. Limbut, C. Thammakhet, P. Thavarungkul, A highly stable oxygen-independent glucose biosensor based on a chitosan-albumin cryogel incorporated with carbon nanotubes and ferrocene. *Sens Actuator B-Chem.* 185(2013) 725-34.

[2] R.M. Kestwal, D. Bagal-Kestwal, B.-H. Chiang, Fenugreek hydrogel–agarose composite entrapped gold nanoparticles for acetylcholinesterase based biosensor for carbamates detection. *Anal Chim Acta.* 886(2015) 143-50.

[3] X. Zhan, W. Tang, W. Dou, G. Zhao, Disposable Immunosensor for Escherichia Coli O157:H7 Based on a Multi-Walled Carbon Nanotube-Sodium Alginate Nanocomposite Film Modified Screen-Printed Carbon Electrode. *Anal Lett.* 46(2013) 2690-704.

[4] A. Fatoni, A. Numnuam, P. Kanatharana, W. Limbut, P. Thavarungkul, A conductive porous structured chitosan-grafted polyaniline cryogel for use as a sialic acid biosensor. *Electrochim Acta.* 130(2014) 296-304.

[5] A. Fatoni, A. Numnuam, P. Kanatharana, W. Limbut, P. Thavarungkul, A novel molecularly imprinted chitosan-acrylamide, graphene, ferrocene composite cryogel biosensor used to detect microalbumin. *Analyst.* 139(2014) 6160-7.

[6] S. Samanman, A. Numnuam, W. Limbut, P. Kanatharana, P. Thavarungkul, Highly-sensitive label-free electrochemical carcinoembryonic antigen immunosensor based on a novel Au nanoparticles–graphene–chitosan nanocomposite cryogel electrode. *Anal Chim Acta.* 853(2015) 521-32.

[7] P. Masawat, A. Harfield, N. Srihirun, A. Namwong, Green Determination of Total Iron in Water by Digital Image Colorimetry. *Anal Lett.* 50(2017) 173-85.

[8] M.B. Lima, S.I.E. Andrade, I.S. Barreto, L.F. Almeida, M.C.U. Araújo, A digital image-based micro-flow-batch analyzer. *Microchem J.* 106(2013) 238-43.

[9] Y. Suzuki, M. Endo, J. Jin, K. IWASE, M. IWATSUKI, Tristimulus colorimetry using a digital still camera and its application to determination of iron and residual chlorine in water samples. *Anal Sci.* 22(2006) 411-4.

[10] C.d.L. Medeiros de Morais, K.M.G. de Lima, A colorimetric microwell method using a desktop scanner for biochemical assays. *Talanta.* 126(2014) 145-50.

[11] Z.J. Smith, K. Chu, A.R. Espenson, M. Rahimzadeh, A. Gryshuk, M. Molinaro, et al., Cell-phone-based platform for biomedical device development and education applications. *PLoS one.* 6(2011) e17150.

[12] O. Mudanyali, S. Dimitrov, U. Sikora, S. Padmanabhan, I. Navruz, A. Ozcan, Integrated

rapid-diagnostic-test reader platform on a cellphone. *Lab on a Chip.* 12(2012) 2678-86.

[13] H. Zhu, U. Sikora, A. Ozcan, Quantum dot enabled detection of Escherichia coli using a cell-phone. *Analyst.* 137(2012) 2541-4.

[14] D. Gallegos, K.D. Long, H. Yu, P.P. Clark, Y. Lin, S. George, et al., Label-free biodetection using a smartphone. *Lab on a Chip.* 13(2013) 2124-32.

[15] J. Wang, Glucose biosensors: 40 years of advances and challenges. *Electroanalysis.* 13(2001) 983.

[16] R. Lowry, Concepts and applications of inferential statistics. (2014).

[17] Z. Machala, B. Tarabova, K. Hensel, E. Spetlikova, L. Sikurova, P. Lukes, Formation of ROS and RNS in Water Electro‐Sprayed through Transient Spark Discharge in Air and their Bactericidal Effects. *Plasma Processes Polym.* 10(2013) 649-59.

[18] C.N. Satterfield, A.H. Bonnell, Interferences in titanium sulfate method for hydrogen peroxide. *Anal Chem.* 27(1955) 1174-5.

[19] T. Eaidkong, R. Mungkarndee, C. Phollookin, G. Tumcharern, M. Sukwattanasinitt, S. Wacharasindhu, Polydiacetylene paper-based colorimetric sensor array for vapor phase detection and identification of volatile organic compounds. *J Mater Chem.* 22(2012) 5970-7.

[20] M. Pávai, E. Orosz, A. Paszternák, Smartphone-Based Extension of the Curcumin/Cellophane pH Sensing Method. *Food Anal Method.* 9(2016) 1046-52.

[21] A. Fatoni, D.W. Dwiasi, D. Hermawan, Alginate cryogel based glucose biosensor. *IOP Conference Series: Materials Science and Engineering.* 107(2016) 012010.

[22] S. Kumar, A. Dwevedi, A.M. Kayastha, Immobilization of soybean (Glycine max) urease on alginate and chitosan beads showing improved stability: Analytical applications. *J Mol Catal B: Enzym.* 58(2009) 138-45.

[23] A. Fatoni, M.D. Anggraeni, D.W. Dwiasi, Simple colorimetric glucose biosensor using chitosan cryogel supporting material. *AIP Conference Proceedings.* 1746(2016) 020029.

[24] I. Taverniers, E. Van Bockstaele, M. De Loose, Analytical Method Validation and Quality Assurance. *Regulations and Quality.* (2008) 743.

[25] I. Kuselman, F. Sherman, Measurement Uncertainty in Chemical Analysis, Springer1999, pp. 252-6.

Figure Captions

Figure. 1 Hollow chitosan cryogel modified biosensing element preparation. The chitosan cryogel was developed inside a micropipette tips, continued by immobilization of GOD, which finally easy to use for glucose detection colorimetrically.

Figure. 2 SEM images of chitosan cryogel surface at x250(a) and x2000(b) showed a porous structure. The SEM image of non-cryogel chitosan showed a flat surface at x250(c) and rough surface without pores at x2000(d).

Figure. 3 Color intensity (red, green, blue) change with the glucose concentration. Inset the example of yellow color produced by resulted hydrogen peroxide reaction with titanium(IV) oxysulfate.

Figure. 4 Enzyme amount effect on the glucose biosensor response. Low blue color intensity was a better response, inverted of formed yellow color visually.

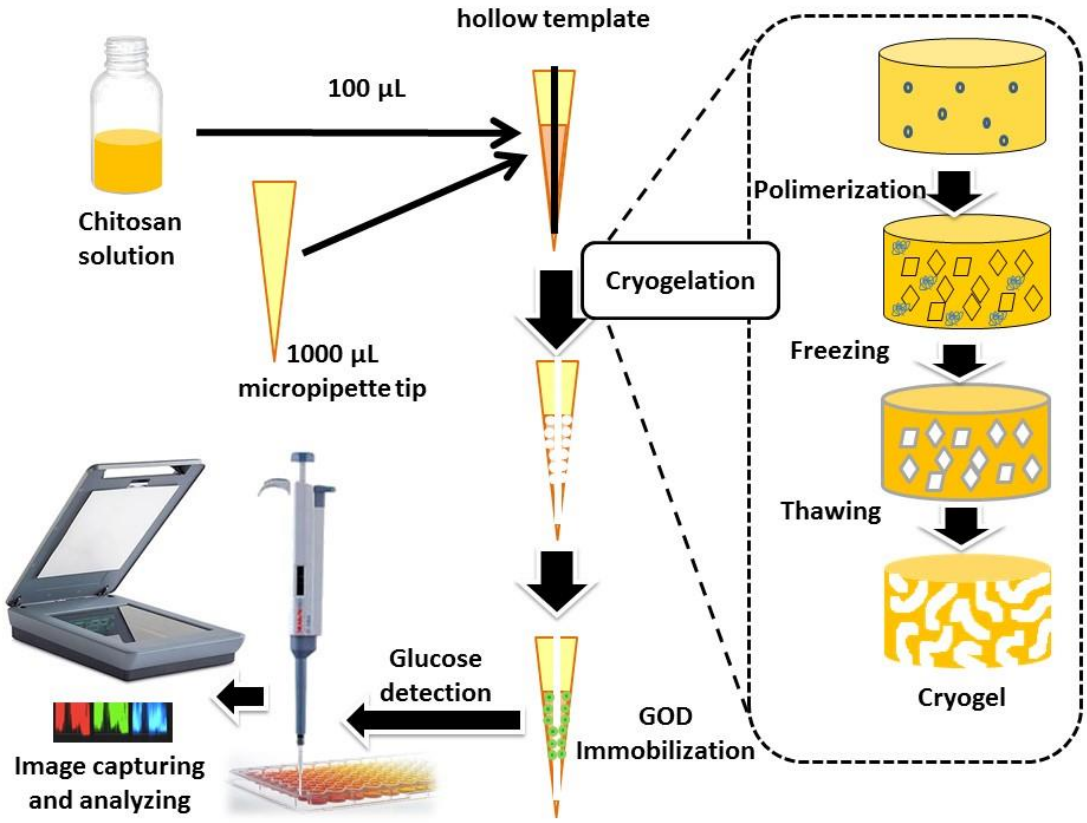
Figure. 5 Enzymatic reaction time effect on the glucose biosensor response. Lower blue color intensity represented a better response of visually yellow color formed.

Figure. 6 Glucose biosensor operational stability performed by uninterrupted analysis of 3.0 mM glucose

Figure. 7 Glucose biosensor responses showed linearly with the glucose concentration of 0.5 to 3.0 mM

Figure. 8 Ascorbic acid and uric acid effect on the glucose biosensor responses.

Figure. 9 Blood glucose analysis using fabricated biosensor (gray bar) compare to the spectrophotometric method of the hospital (dark blue bar).



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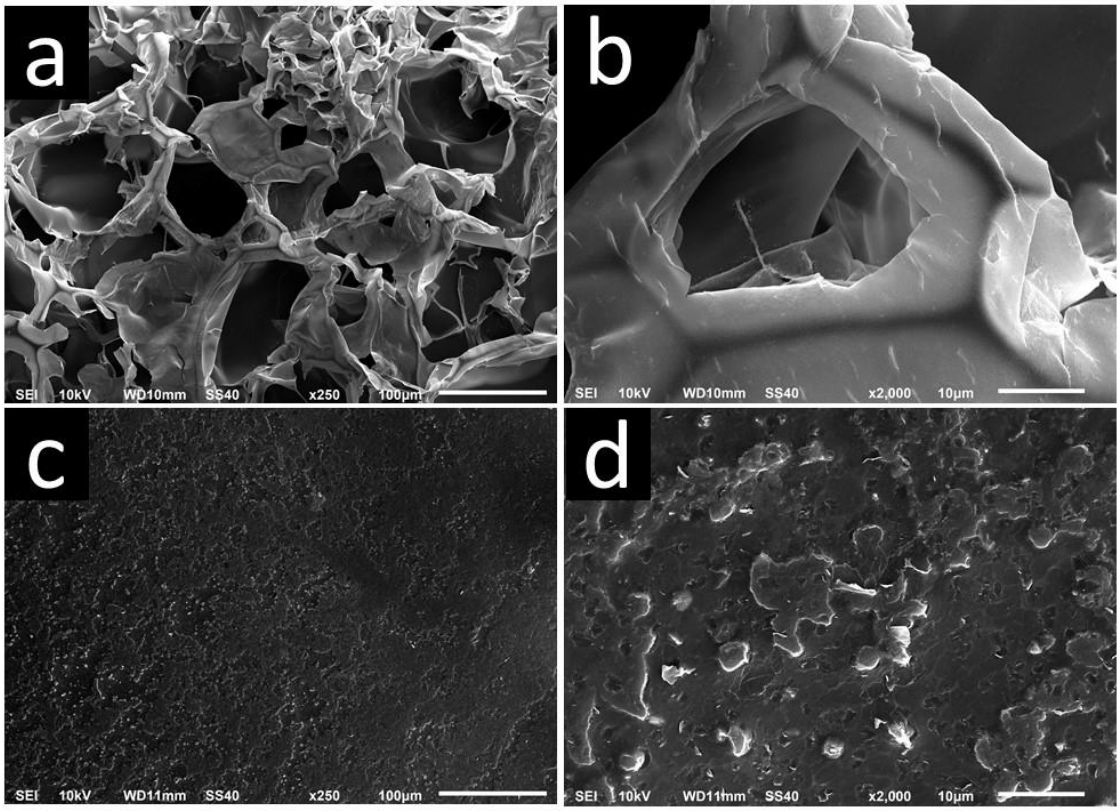

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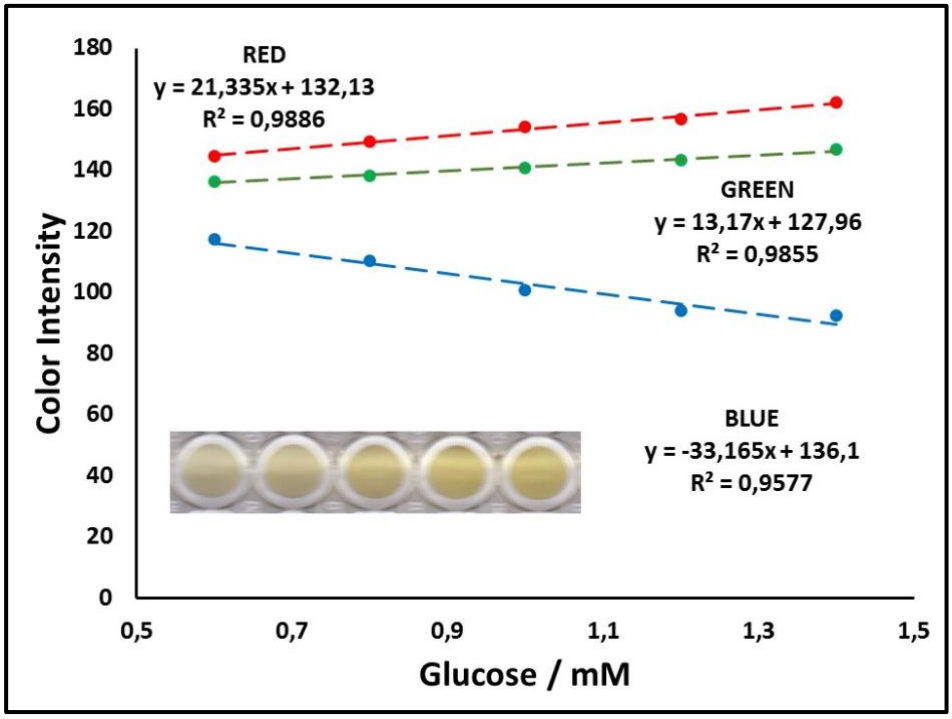

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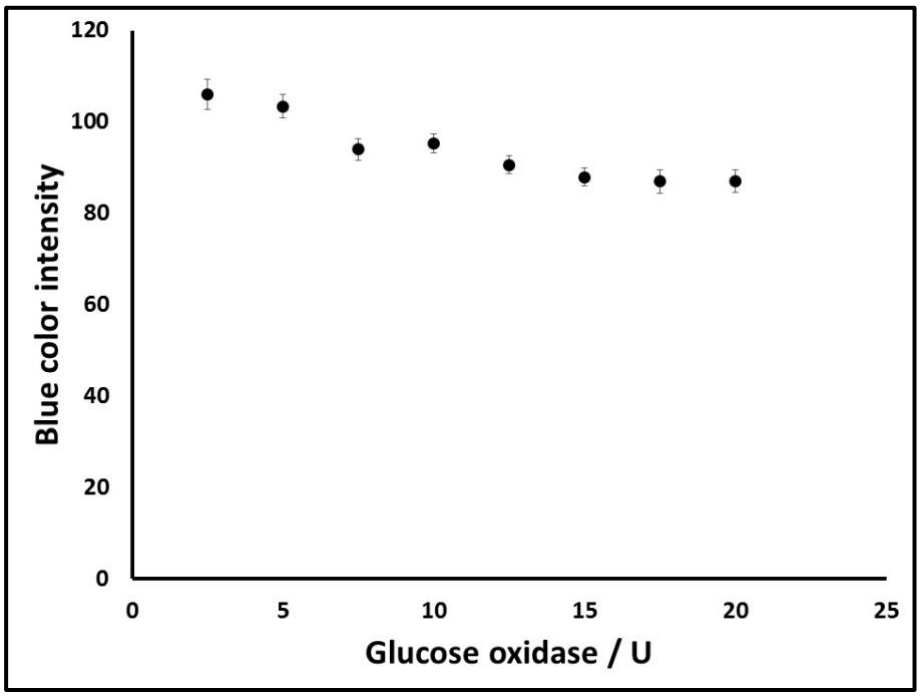

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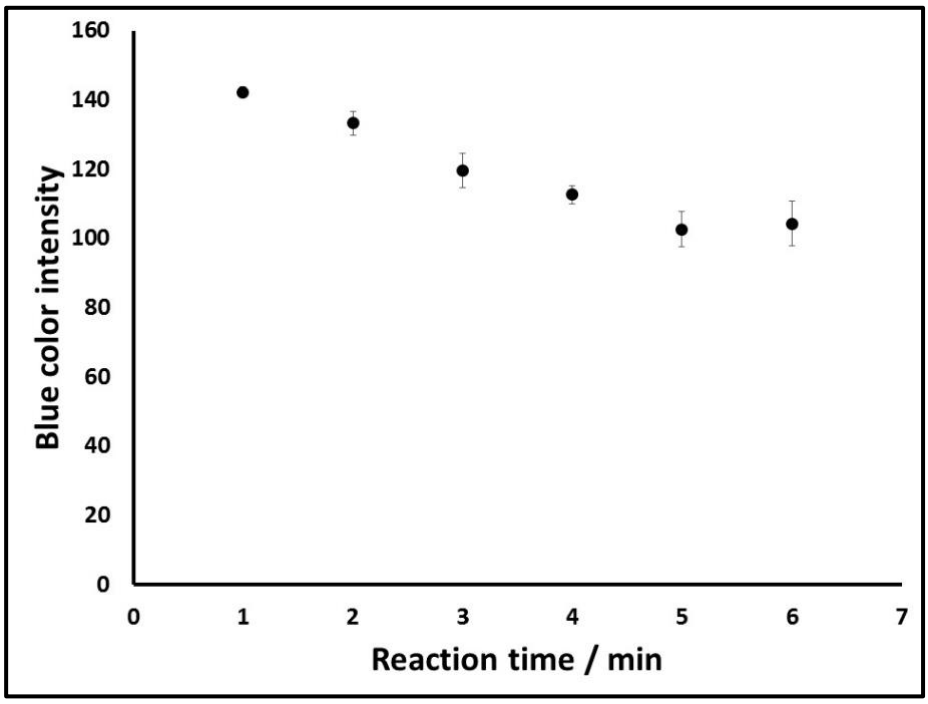

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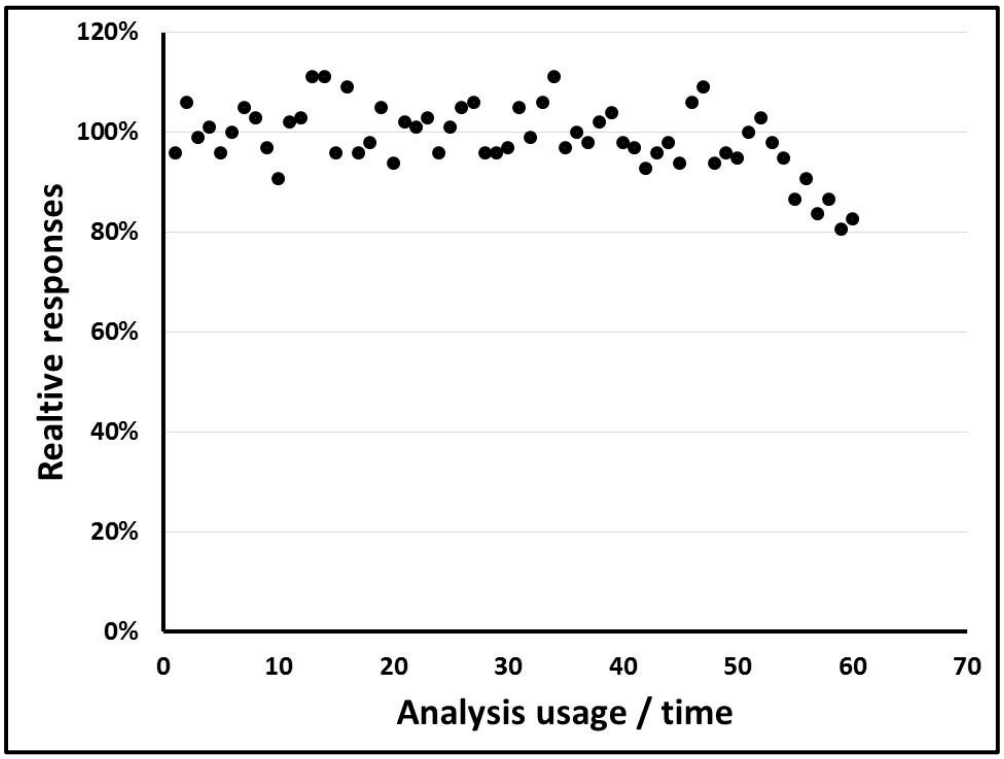

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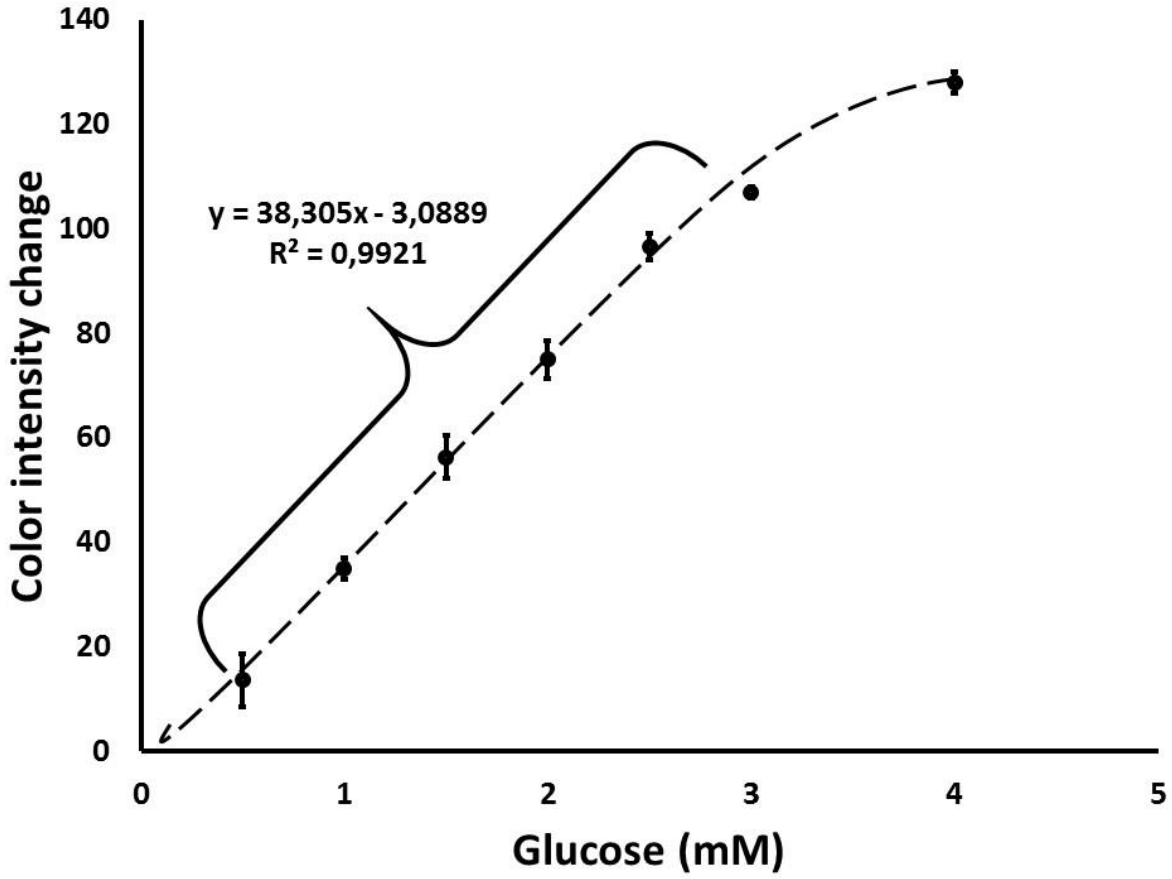

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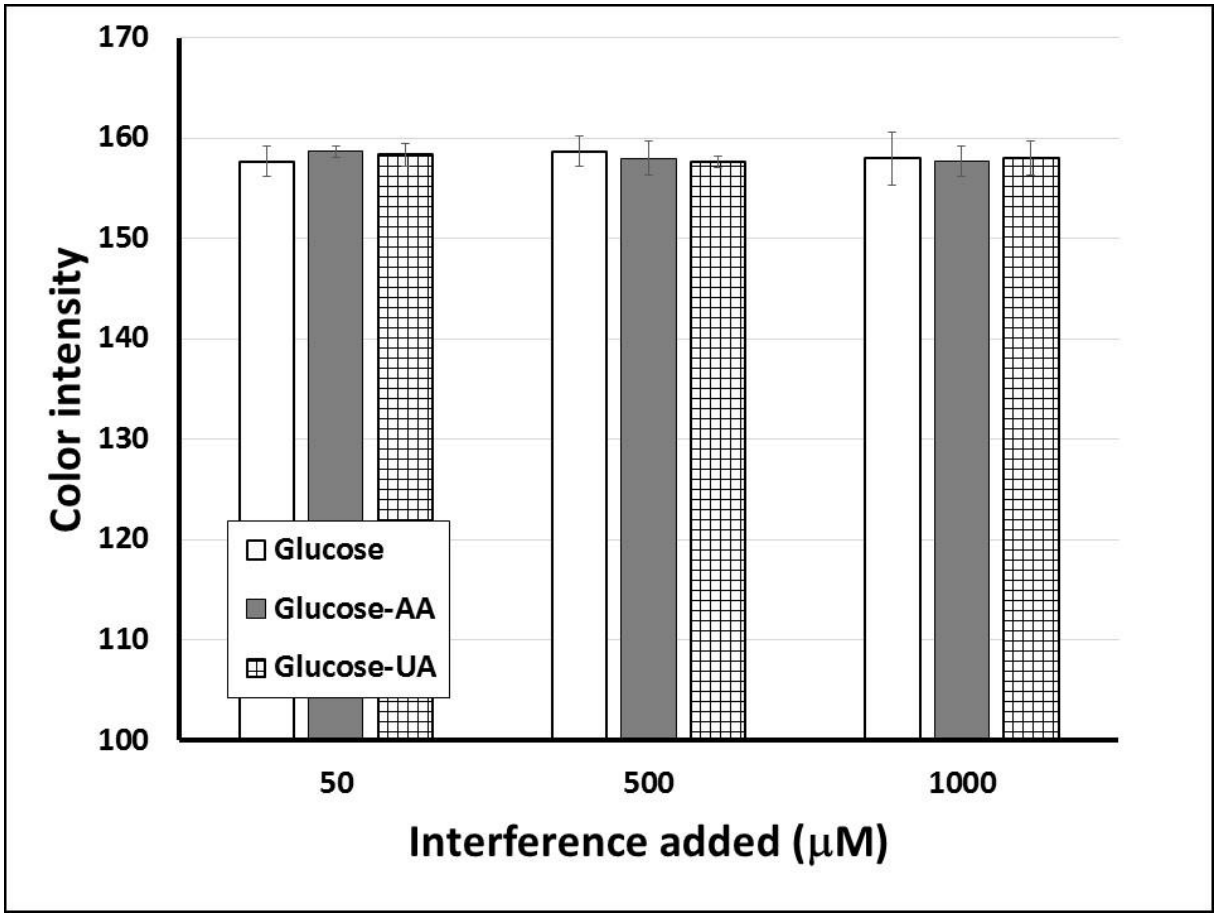

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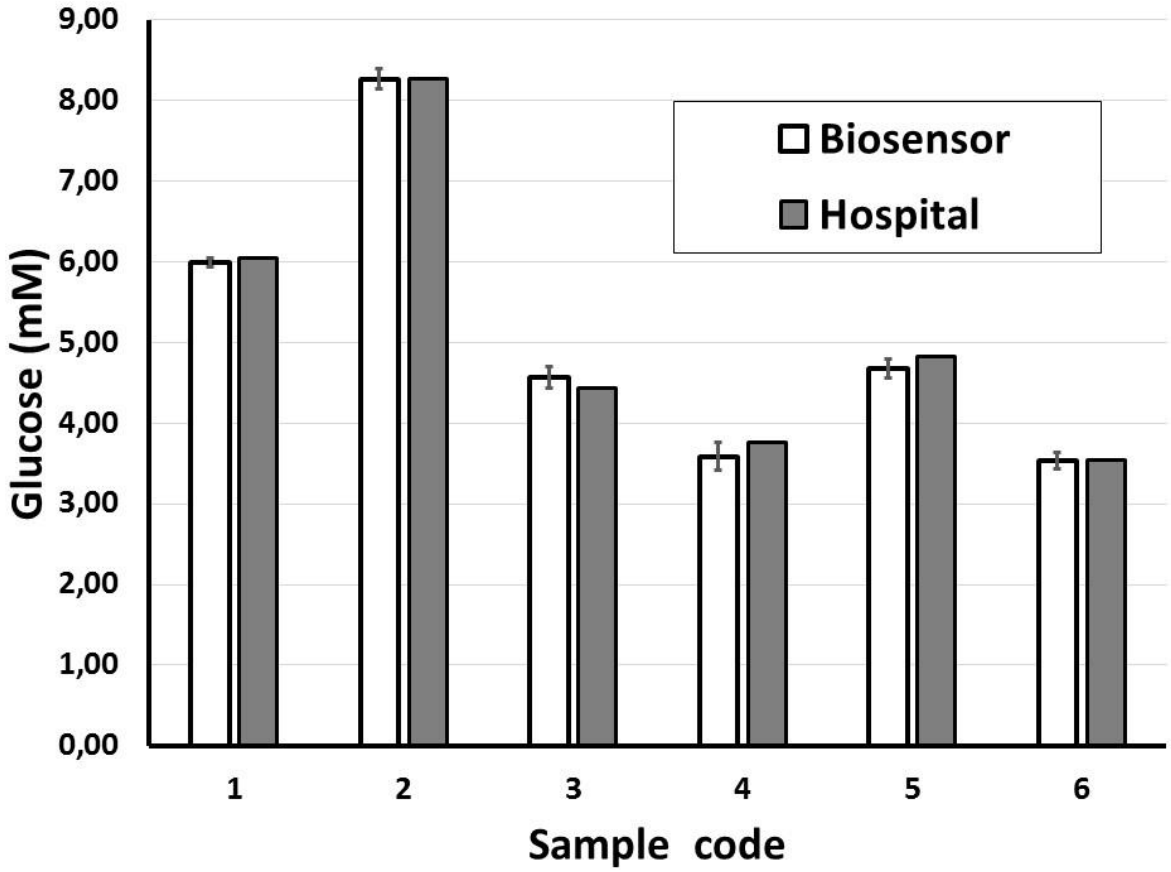

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