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PII: S0141-8130(18)33018-6
DOI: doi:10.1016/j.ijbiomac.2018.08.163
Reference: BIOMAC 10407

To appear in: *International Journal of Biological Macromolecules*

Received date: 18 June 2018
Revised date: 11 August 2018
Accepted date: 27 August 2018

Please cite this article as: Khodabakhsh Rashidi, Majid Mahmoudi, Ghobad Mohammadi, Mohammad Mahdi Zangeneh, Shahla Korani, Hector C. Goicoechea, Hui-Wen Gu, Ali R. Jalalvand , Simultaneous co-immobilization of three enzymes onto a modified glassy carbon electrode to fabricate a high-performance amperometric biosensor for determination of total cholesterol. Biomac (2018), doi:10.1016/j.ijbiomac.2018.08.163

Simultaneous co-immobilization of three enzymes onto a modified glassy carbon electrode to fabricate a high-performance amperometric biosensor for determination of total cholesterol

Khodabakhsh Rashidi[a], Majid Mahmoudi[a], Ghobad Mohammadi[b], Mohammad Mahdi Zangeneh[c,d], Shahla Korani[a], Hector C. Goicoechea[e], Hui-Wen Gu[f], Ali R. Jalalvand[a,*]

[a]*Research Center of Oils and Fats, Kermanshah University of Medical Sciences, Kermanshah, Iran*
[b]*Department of Pharmaceutics, School of Pharmacy, Kermanshah University of Medical Sciences, Kermanshah, Iran*
[c]*Department of Clinical Science, Faculty of Veterinary Medicine, Razi University, Kermanshah, Iran*
[d]*Pharmaceutical Sciences Research Center, Faculty of Pharmacy, Kermanshah University of Medical Sciences, Kermanshah, Iran*
[e]*Laboratorio de Desarrollo Analítico y Quimiometría (LADAQ), C_atedra de Química Analítica I, Universidad Nacional del Litoral, Ciudad Universitaria, CC 242 (S3000ZAA), Santa Fe, Argentina*
[f]*College of Chemistry and Environmental Engineering, Yangtze University, Jingzhou 434023, China*

**Corresponding Author: Tel.: +988334302345; Fax: +988334279745; E-mail Address:* ali.jalalvand1984@gmail.com (A.-R. Jalalvand)

Abstract

In this work, we have fabricated a novel amperometric cholesterol (CHO) biosensor because of the importance of determination of CHO levels in blood which is an important parameter for diagnosis and prevention of disease. To achieve this goal, cholesterol oxidase, cholesterol esterase and horseradish peroxidase were simultaneously co-immobilized onto a glassy carbon electrode (GCE) modified with gold nanoparticles/chitin-ionic liquid/ poly(3,4-ethylenedioxypyrrole)/graphene-multiwalled carbon nanotubes-1,1′-ferrocenedicarboxylic acid-ionic liquid. Modifications applied to the bare GCE were characterized by cyclic voltammetry, electrochemical impedance spectroscopy and scanning electron microscopy. The biosensor detected CHO in linear ranges of 0.1-25 μM and 25-950 μM with a detection limit of 0.07 μM. The sensitivity of the biosensor was estimated to be 6.6 μA μM^{-1} cm^{-2}, its response time was less than 5 s and Michaelis-Menten constant was calculated to be 0.12 μM. Results obtained in this study revealed that the biosensor was selective, sensitive, stable, repeatable and reproducible. Finally, the biosensor was successfully applied to the determination of CHO levels in rats plasma.

Keywords: Cholesterol; Biosensor; Rats plasma.

1. Introduction

Cholesterol (CHO, Fig. S1) is a fat-like substance and all cells of the body contain CHO. CHO is needed in the body for the synthesis of vitamin D, hormones and some substances which help to the digestion of foods. The body is able to make all the needed CHO however, CHO can also be found in some foods. The CHO in blood stream could be found as small packages called lipoproteins which travel through bloodstream. Determination of CHO level in blood is very important in clinical point of view because of its importance in the diagnosis and prevention of disease [1,2]. Generally, the total CHO concentration in blood of a healthy person must be less than 200 mg/dL (<5.17 mM), the borderline high is 200-239 mg/dL (5.17-6.18 mM) and values higher than 240 mg/dL (≥6.21mM) are defined as the high value [3]. Determination of CHO concentration in blood is a routine analysis in medical diagnosis. High CHO levels in bloodstream are strongly correlated with arteriosclerosis and coronary heart disease [4,5]. There are several methods such as calorimetric method, liquid chromatography, gas chromatography, high performance liquid chromatography, spectrophotometric methods and thermistor based assays which are applied to the determination of CHO [6-11]. These methods are accurate but suffer from some limitations such as cost, time, instrumentation, skilled operators and standardization difficulties. Therefore, developing efficient analytical methods for determination of CHO is highly demanded and among the existing methods enzymatic procedures are gaining widespread attention.

Enzymatic biosensors which have immobilized enzymes as their bio-recognition element are gaining widespread attentions by the researchers because of their capabilities from research and application points of view [12-14]. Immobilization of the enzyme onto the biosensor surface could be performed by entrapment, adsorption, cross-linking and covalent attachment [15-18]. Among the mentioned approaches, cross-linking approach is preferable because of the limitation of physical adsorption by some problems such as enzyme leaching from the biosensor surface [19]. In CHO biosensors, cholesterol oxidase (ChO) is the most frequently used biosensing element which causes directly determination of CHO concentration. ChO in the presence of

oxygen catalyzes the oxidation of CHO to hydrogen peroxide and cholest-4-en-3-one according to the following reaction [20]:

$$CHO + O_2 \rightarrow \text{cholest-4-en-3-one} + \text{Hydrogen Peroxide} \quad \textbf{(1)}$$

By applying a suitable potential to the system, electrochemical oxidation of hydrogen peroxide is occurred which its current is directly correlated with CHO concentration. The most important problem with this detection is the interfering effects of interferences such as ascorbic acid. This problem can be tackled by using more than one enzyme as recognition element such as combination of two or three enzymes which causes more selectivity for the CHO [3]. CHO in blood serum could be found in the form of ester therefore, for its determination it must be hydrolyzed which could be performed by cholesterol esterase (ChE). As a result, by combination of ChO and ChE, the esterified CHO is hydrolyzed by CHE and subsequently will be oxidized by ChO to produce cholest-4-en-3-one and hydrogen peroxide. As reported by the other researchers, horseradish peroxidase (HRP) can be co-immobilized with ChO and ChE onto the biosensor surface to reduce interference [21]. HRP contains ferroheme/ferriheme pair as redox center and conversion of its reduced state to its oxidized state is performed through direct electron transfer. There is a heme group present in outer region of HRP in its 3D configuration which enhances the direct electron transfer between redox center and electrode [22]. Regeneration of HRP is performed by direct electron transfer according to following reactions:

$$H_2O_2 + 2H^+ + HRP_{Red} \rightarrow 2H_2O + HRP_{Oxd.} \quad \textbf{(2)}$$

$$HRP_{Oxd.} + 2e^- \leftrightarrow HRP_{Red.}$$

Another important outcome which could be obtained by immobilization of the enzymes onto the biosensor surface is reducing the Michaelis constant (??$_{??}$) and enhancing bimolecular rate constant which improves performance of the biosensor [23-26].

In fabricating electrochemical sensors and biosensors, the bare glassy carbon electrode (GCE) is usually modified with different materials which can help to improve analytical characteristics of the developed sensors and biosensors [27-39]. Many researchers have reported that modification of the electrode can help to decrease

the overpotential applied to the amperometric detection of CHO [40-49]. Therefore, to achieve this goal, we have modified the bare GCE.

Graphene (Gr) is a monolayer of bonded carbon atoms (sp^2) with versatile electronic, mechanical and thermal properties and owing to its interesting physicochemical properties has found a lot of applications in different fields of research especially in constructing biosensors [50-52]. On the other hand, carbon nanotubes (CNTs) as rolled graphene sheets also show high electron transfer rate, high surface area, minimization of the surface fouling, high stability and excellent electrocatalytic properties [53,54]. Incorporation of Gr with CNTs yields a hybrid material as a versatile platform for the electrocatalytic applications. Ionic liquids (ILs) due to high chemical and electrochemical stability and ionic conductivity have obtained a lot of applications in constructing electrochemical sensors and biosensors [55]. Advantages of combination of ILs with CNTs or Gr to obtain composite gels have been already exploited [56-59]. Ferrocene and its derivatives such as 1,1′-ferrocenedicarboxylic acid (Fr) due to their good stability in both oxidized and reduced forms and stability in solution, fast and independent pH response and fast electron transfer have been classified within the most successful mediators [60,61]. Electron-rich polymers such as poly(3,4-ethylenedioxypyrrole) (PEDOP) due to important properties such as high conductivity and mechanical stability are every useful for forming stable conducting polymer films which have a lot of applications in chemical sensing. Chitin (Ch) is a natural and biocompatible biopolymer which has been attracted attention of electrochemists due to its important properties such as good film forming ability, nontoxicity and high mechanical strength [3]. It has been proven that some mechanisms such as chelation, electrostatic attraction and ion exchange enables Ch to accumulate metal ions [3]. To improve the properties of Ch, combination of Ch with other materials such as ILs has been reported [3]. Electrodeposition is a controllable and green technique for the synthesis of metallic nanoparticles (NPs) where characteristics of the NPs such as size, shape and density can be controlled by instrumental parameters.

Sesame oil with a distinctive nutty aroma and taste is an edible vegetable oil derived from sesame seeds [62]. Besides being used as a cooking oil, it is used as a flavour enhancer [62]. Sesame oil with different

biomedical actions such as an effect on lipid metabolism in human and animal can affect the CHO levels in blood stream as reported by the other researchers [63].

In this work, we are going to fabricate a novel and interesting amperometric CHO biosensor based on co-immobilization of cholesterol oxidase (ChO), cholesterol esterase (ChE) and horseradish peroxidase (HRP) onto Au NPs/Ch-IL/PEDOP/Gr-MWCNTs-Fr-IL/GCE by cross-linking enzymes and Ch through addition of glutaraldehyde. After characterization of the modification steps, the biosensor will be analytically characterized and finally, it will be applied to verification of the sesame oil effects on CHO levels in blood of 20 classified rats fed programmed diets during five weeks. Schematic representation of the steps of this study is showing in Scheme 1. On the whole, we are going to fabricate a novel and high-performance amperometric biosensor for determination of CHO and for verifying its performance, it will be applied to investigate the effects of sesame oil on CHO levels in rats blood.

Scheme 1

2. Experimental

2.1. Chemicals, solutions and animals

ChO, ChE, CHO, HRP, hydrogen peroxide (H_2O_2), 1-ethyl-3-methylimidazolium bis(trifluoromethylsulfonyl) imide (IL), isopropanol, Na_2SO_4, Ch, Triton X-100, Fr, 3,4-ethylenedioxypyrrole (EDOP), glutaraldehyde, potassium ferrocyanide, potassium ferriccyanide, gold (III) tetrachloride ($HAuCl_4$), dimethylformamide (DMF), acetic acid, sodium phosphate monobasic (NaH_2PO_4), sodium phosphate dibasic (Na_2HPO_4), ethanol, phosphoric acid (H_3PO_4) and sodium hydroxide (NaOH) were purchased from Sigma. The Gr was purchased from PubChem. The MWCNTs were purchased from Ionic Liquid Technologies. The other chemicals used in this study were purchased from well-known sources and used as received. All the solutions used in this work were prepared in doubly distilled water (DDW). Highly pure N_2 was applied for deaeration. Sesame oil was freshly prepared from sesame seeds. Twenty Sprague-Dawley male rats weighing 180-215 g were purchased from school of medicine of Kermanshah University of Medical Sciences, Kermanshah, Iran.

NaH_2PO_4, and Na_2HPO_4 were used to prepare a phosphate buffered solution (PBS, 0.05 M) and H_3PO_4 and NaOH were used to adjust its pH at 7.0. Step-by-step characterizations of the modifications were

performed by a redox probe solution, $[Fe(CN)_6]^{3-/4-}$, with a concentration level of 0.05 M prepared in the PBS (0.05 M, pH 7.0). The Gr-MWCNTs-Fr-IL was prepared by the addition of 5.0 µL IL to 1.0 mL DMF containing 2.0 mg MWCNTs, 2.0 mg Gr and 1.0 mg Fr and ultrasonicated for 30.0 min. A EDOP (0.01 M) solution was prepared in 0.1 M $LiClO_4$. A stock solution of Ch (0.2% w/v) was prepared in acetic acid by ultrasonication during 1.0 hour. The Ch-IL was prepared by adding 1.0 µL IL into 1.0 mL Ch (0.2%). A solution of $HAuCl_4$ (0.5 mM) was prepared in Na_2SO_4 (0.2 M). A stock solution of CHO (0.1 M) was prepared in the PBS (0.05 M, pH 7.0) containing 1% Triton X-100 (v/v) and 20% (v/v) isopropanol, and stored at -23 ° C. The CHO working solutions were prepared from its stock solution by applying appropriate dilutions. ChO (40 U/mL), ChE (40 U/mL) and HRP (40 U/mL) were dissolved in the PBS (0.05 M, pH 7.0) and stored at -23 °C.

2.2. Instruments and softwares

All the electrochemical data reported in this study were collected by an Autolab PGSTAT302N-high performance controlled by the NOVA software. The results of this study were compared with those of obtained by a diagnostic laboratory where the serum samples were analyzed by Total Cholesterol Assay Kits. The SEM images were captured by a KYKY-EM 3200 scanning electron microscope. A rotating disc electrode with a glassy carbon disk as working electrode, a Pt wire as counter electrode and an Ag/AgCl electrode as reference electrode were purchased from Metrohm Company. A Sigma centrifuge was used to centrifuge the blood samples. A JENWAY-3345 pH-meter equipped with a combined glass electrode was applied to pH adjustments. The recorded electrochemical data was transferred to MATLAB (Version 7.14, MathWorks, Inc.) environment and smoothed when necessary. All the computations were performed on a DELL XPS laptop (L502X).

2.3. Preparation of the biosensor

The GCE was well polished in an alumina slurry by a silky pad and then, rinsed with DDW and ultrasonicated in ethanol for 30.0 min and left to be dried at room temperature. The Gr-MWCNTs-Fr-IL/GCE was fabricated by dropping 10.0 µL of Gr-MWCNTs-Fr-IL solution onto the surface of the cleaned GCE.

Electropolymerization of EDOP onto the surface of Gr-MWCNTs-Fr-IL/GCE to fabricate PEDOP/Gr-MWCNTs-Fr-IL/GCE was performed by cyclic voltammetry (CV) via immersing the Gr-MWCNTs-Fr-IL/GCE into $LiClO_4$ solution (0.1 M) containing EDOP under applying 40 cycles. Ch-IL/PEDOP/Gr-MWCNTs-Fr-IL/GCE was fabricated by dropping 10 μL Ch-IL onto the surface of PEDOP/Gr-MWCNTs-Fr-IL/GCE. Then, Au NPs were electrochemically deposited onto the surface of Ch-IL/PEDOP/Gr-MWCNTs-Fr-IL/GCE via CV by immersing it into $HAuCl_4$ (0.5 mM) prepared in Na_2SO_4 (0.2 M) under applying 6 cycles and scanning potential from 0 to -0.8 V. Finally, Au NPs/Ch-IL/PEDOP/Gr-MWCNTs-Fr-IL/GCE was immersed into glutaraldehyde solution for 2 h and then, 10 μL of a solution containing ChO (40 U/mL), ChE (40 U/mL) and HRP (40 U/mL) was dropped onto its surface and left to be dried at room temperature and kept in a refrigerator until analysis time.

2.4. Animals

Twenty Sprague-Dawley male rats were purchased from the animal house of Kermanshah University of Medical Sciences, Kermanshah, Iran where skilled operators maintained and fed the rats. At the end of period, skilled operators drawn the blood samples by cardiac puncture and analyses of the blood samples were performed in Research Center of Oils and Fats of Kermanshah University of Medical Sciences, Kermanshah, Iran. Twenty Sprague-Dawley male rats weighing 180-215 g were divided into four groups including control group, group 1, group 2 and group 3 where each group had five rats. The rats were maintained in individual cages under controlled conditions such as temperature, humidity and illumination and allowed free access to food and water for five weeks. The control group was fed mouse pellets, group 1 was fed mouse pellets+10% (w/w) sesame oil, group 2 was fed a high fat diet consisting of mouse pellets+20% (w/w) fat and group 3 was fed mouse pellets+20% (w/w) fat+10% (w/w) sesame oil. The corpses of the rats at the end of period and after cardiac puncture are showing in Fig. 1.

Figure 1

2.5. Preparation of serum samples

Blood samples obtained from cardiac punctures were collected in tubes and centrifuged at 4500 rpm for 20 min. Then, plasma samples were separated and treated with the PBS (0.05 M, pH 7.0) containing 1% Triton X-100 (v/v) and 20% (v/v) isopropanol. Finally, the prepared plasma samples were poured into covered tubes and kept in a refrigerator at – 23 °C until analysis time.

3. Results and discussion

3.1. Morphological characterizations

Morphological characterization as an important and effective evidence could be applied to the surface of the biosensor by SEM to confirm the modification steps. The SEM images captured from the surface of the modified electrodes are showing in Fig. 2. Fig. 2A shows the SEM image captured from the surface of Gr-MWCNTs-Fr-IL/GCE which confirms that Gr sheets and tubes of MWCNTs integrated with Fr have been attached to the surface of the GCE. Fig. 2B confirms that the PEDOP has formed a layer on the surface of Gr-MWCNTs-Fr-IL/GCE. After modification of PEDOP/Gr-MWCNTs-Fr-IL/GCE with Ch-IL, a new layer formed on the surface of the electrode which can be clearly observed in Fig. 2C. Fig. 2D shows the Au NPs deposited onto the surface of Ch-IL/PEDOP/Gr-MWCNTs-Fr-IL/GCE which also confirms that the electrodeposition is a reliable method for synthesizing NPs. Fig. 2E shows the SEM image captured from the biosensor surface after co-immobilizing ChO, ChE and HRP which confirms the presence of the enzymes at the biosensor surface.

Figure 2

3.2. Electrochemical characterizations

The CV is an effective electrochemical technique which could be applied to monitor the modifications applied to the electrode surface. Therefore, modifications applied to the bare GCE to fabricate the biosensor were characterized by CV. The CVs recorded by immersing different electrodes into the redox probe solution, $[Fe(CN)_6]^{3-/4-}$, with a concentration level of 0.05 M prepared in the PBS (0.05 M, pH 7.0) are showing in Fig. S2A. As can be seen, the bare GCE showed a well-defined CV (curve a) which its peak currents were increased

and its peaks separation ($\Delta E=E_{pc}-E_{pa}$) was decreased after modifying with Gr-MWCNTs-IL (curve b). The CV of the bare GCE was further improved (enhancing peak currents and decreasing ΔE) by the addition of Fr to Gr-MWCNTs-IL (curve c) which manifested larger surface area and more facile electron transfer for Gr-MWCNTs-Fr-IL/GCE. When EDOP was electrochemically polymerized onto the surface of Gr-MWCNTs-Fr-IL/GCE, the peak currents were enhanced (curve d) which shows the role of conducting PEDOP in modification process. After drop-casting of Ch-IL onto the surface of PEDOP/Gr-MWCNTs-Fr-IL/GCE, the peak currents were decreased and ΔE was increased (curve e) which confirmed that Ch was able to decrease the rate of charge transfer in some extent. As can be seen, the best performance was observed when Au NPs were electrochemically synthesized at the surface of Ch-IL/PEDOP/Gr-MWCNTs-Fr-IL/GCE (curve f) which confirmed the great role of NPs in expanding the electroactive surface area and fastening the rate of electron transfer.

Electrochemical impedance spectroscopy (EIS) is a valuable electrochemical technique which could be applied to monitor the modification steps. The Nyquist plot of the EIS includes a semicircle portion at high frequencies which its diameter is proportional to the resistance of charge transfer (R_{ct}) at the electrode surface and could be used to describe the interface properties of the electrode surface. The Nyquist plot of EIS also includes a linear portion which is related to the diffusion process. Therefore, according to the information described above, we have used the EIS method to monitor the modifications applied to the bare GCE to fabricate the CHO biosensor. The EIS measurements were performed in an electrochemical cell containing the redox probe solution, $[Fe(CN)_6]^{3-/4-}$, with a concentration level of 0.05 M prepared in the PBS (0.05 M, pH 7.0) where the bare or modified GCE acted as working electrode, a Pt wire as counter electrode and an Ag/AgCl as reference electrode. The EIS curves were fitted by fit and simulation command provided by the NOVA software to obtain the best circuit (inset of Fig. S2B) and then, their R_{ct} values were calculated. The Nyquist plots of the bare GCE (curve a), Gr-MWCNTs-IL/GCE (curve b), Gr-MWCNTs-Fr-IL/GCE (curve c), PEDOP/Gr-MWCNTs-Fr-IL/GCE (curve d), Ch-IL/PEDOP/Gr-MWCNTs-Fr-IL/GCE (curve e) and Au NPs/Ch-IL/PEDOP/Gr-MWCNTs-Fr-IL/GCE (curve f) are showing in Fig. S2B. After fitting the EIS curve of

the bare GCE, its R_{ct} value was calculated to be 450 Ω. After modifying the bare GCE with Gr-MWCNTs-IL, its R_{ct} value was decreased (244 Ω) which confirmed this layer enhanced the rate of electron transfer at the electrode surface. The presence of Fr in Gr-MWCNTs-IL on the electrode surface decreased the R_{ct} value (163 Ω) which manifested that the Gr-MWCNTs-Fr-IL was acted better than Gr-MWCNTs-IL. After electropolymerization of EDOP at the surface of Gr-MWCNTs-Fr-IL/GCE, the R_{ct} value was further decreased (146 Ω) which confirmed a more facile electron transfer was occurred at the electrode surface due to the presence of PEDOP. By drop-casting of Ch-IL onto the surface of PEDOP/Gr-MWCNTs-Fr-IL/GCE, the diameter of the semicircle was increased (219 Ω) which suggested a slower electron transfer at the electrode surface due to the presence of Ch. Electrodeposition of NPs at the Ch-IL/PEDOP/Gr-MWCNTs-Fr-IL/GCE surface caused a significant acceleration in rate of electron transfer as is obvious from R_{ct} value which was calculated to be 39 Ω. NPs accelerate the redox probe reaction at the electrode surface by opening a lot of new conduction pathways to facilitate electron transfer between electrode surface and electrolyte solution.

3.3. Performance evaluation of different modified electrodes toward hydrogen peroxide reduction

All the CVs showed in this section were recorded in the absence of O_2 by deaeration of the electrolyte solution with the help of highly pure N_2 for 15 min. Fig. 3A and Fig. 3B show the typical CVs recorded by different electrodes in the absence and presence of H_2O_2, respectively. As can be seen, all the electrodes examined in the absence of H_2O_2 did not show any peak at -0.15 V but, their response in the presence of H_2O_2 appeared at -0.1 V which was related to the reduction of H_2O_2. Among the examined electrodes, Au NPs/Ch-IL/PEDOP/Gr-MWCNTs-Fr-IL/GCE exhibited the most sensitive response (Fig. 3B, curve f) therefore, it can be deduced that applying good modification steps to fabricate the Au NPs/Ch-IL/PEDOP/Gr-MWCNTs-Fr-IL/GCE have significantly promoted the electron transfer of H_2O_2 reduction. The inset of Fig. 3B shows the CV responses of Au NPs/Ch-IL/PEDOP/Gr-MWCNTs-Fr-IL/GCE upon increasing concentration of H_2O_2 which confirmed good performance of the modified electrode towards hydrogen peroxide reduction.

Figure 3

3.4. Analytical performance of the fabricated biosensor

Since amperometry under stirred conditions has a much higher current sensitivity than CV, it was used to build the calibration curve. Amperometric measurements were performed in the absence of O_2 by deaeration of the electrolyte solution with the help of highly pure N_2 for 15 min. Fig. 4A displays amperometric response of ChOChEHRP/Au NPs/Ch-IL/PEDOP/Gr-MWCNTs-Fr-IL/GCE against successive injections of CHO into continuously stirred PBS (0.05 M, pH 7) at an applied potential of -0.15 V. To build the calibration curve, the current values were regressed on concentration values as shown in Fig. 4B. The biosensor exhibited two linear ranges of response to CHO, in the concentration ranges of 0.1-25 μM and 25-950 μM with correlation coefficients of 0.9863 and 0.993, respectively. The calibration curve slope was changed at the CHO concentration of 25 μM. Upon successive additions of CHO, the concentration of Triton X-100 was also increased in the solution which consequently caused a decrease in the biosensor response as it was suggested by other researchers [64,65]. The sensitivity of the biosensor was 6.6 μA μM^{-1} cm^{-2} (slope of the calibration curve) and the limit of detection (LOD=3S_b/m, where S_b is the standard deviation of the blank and m is the slope of the calibration curve) was calculated to be 0.07 μM. The current of the fabricated biosensor increased immediately after the addition of CHO and reached a steady value. The average time required to reach 90% of the saturation current value (response time, RT) was determined as less than 5 s which is a short RT.

Figure 4

3.5. Interference study

The influence of interfering species such as glucose (GL, 100 μM), ascorbic acid (AA, 200 μM), uric acid (UA, 150 μM), urea (UR, 250 μM) and bilirubin (BL, 250 μM) which may coexisted with CHO in blood serum was examined by investigating their effect on the amperometric response of CHO (20 μM) and the results are showing in Fig. 5A. As can be seen, there was no any interference from glucose (GL, 100 μM), ascorbic acid (AA, 200 μM), uric acid (UA, 150 μM) and bilirubin (BL, 250 μM). The amperometric detection of H_2O_2 is generally carried out by following the oxidation current responses at positive potentials (> 0.5 V) therefore, such a good selectivity observed for the fabricated biosensor in this study against coexisting electroactive species owes to monitoring the reduction current of H_2O_2 at a far negative potential (-0.15 V) which caused

these compounds not to interfere with the determination of CHO. Therefore, studies performed in this section confirmed a good selectivity for the fabricated biosensor.

Figure 5

3.6. Stability, repeatability and reproducibility of the biosensor

Long-term stability of a biosensor is an important parameter which must be reported to clarify its capability for practical purposes therefore, stability of the biosensor was evaluated by weekly measurement of its response during six weeks and the results are showing in Fig. 5B. As can be seen, the biosensor retained 94.5% of its original response after six weeks which confirms a high stability for the biosensor. This result confirmed that a suitable platform with a good biocompatibility has been provided which maintains the biological activity of the immobilized enzymes. Repeatability of the proposed biosensor was also evaluated by applying it for eight times during a day to determine 100 μM CHO and a satisfied relative standard deviation (RSD) of 2.35% was found which confirmed a good repeatability for the biosensor. The reproducibility of the biosensor was also verified by recording the responses of ten individual biosensors immersed into a solution containing 100 μM CHO and a good RSD value of 3.1% was obtained which confirmed a good reproducibility for the biosensor. The information mentioned above, confirmed that the biosensor response towards CHO determination was stable, repeatable and reproducible.

The apparent Michaelis-Menten constant (K_m) which gives an indication of the enzyme-substrate kinetics for the biosensors could be calculated from the electrochemical version of the Lineweaver-Burk equation (Eq. (3)):

$$\frac{1}{i_{ss}} = \frac{1}{i_{max}} + \frac{k_m}{i_{max}} C \qquad \textbf{(3)}$$

where i_{ss} is the steady state current after the addition of substrate, C is the bulk concentration of the substrate, and i_{max} is the maximum current measured under saturated substrate condition. The K_m value of the CHO biosensor was determined by plotting the steady state amperometric response ($1/i_{ss}$) vs. $1/C$ (not shown) and was found to be 0.12 μM.

3.7. Comparison with previous biosensors reported in literature

The results obtained by the fabricated biosensor in this study were compared with those of previous works reported in the literature and the results are presented in Table S1. As can be seen, the proposed biosensor showed better results over the most of the reported sensors so far.

3.8. Application of the fabricated biosensor to the analysis of blood serum samples and its validation

Generally, after developing a new analytical method and validating its performance for the analysis of both synthetic and real samples, its performance is compared with a reference method to verify its capability for the analysis of real samples. Then, the newly developed analytical method could be suggested as a reliable method for using by the others. Regarding this important step, we have also applied total cholesterol assay kits to the analysis of serum samples towards CHO determination and its results are presented in Table S2. Final concentration of CHO in serum samples were calculated by multiplying an appropriate dilution factor of 1000. As can be seen, there is a good agreement between the results obtained by the proposed biosensor (amperograms recorded by the biosensor are showing in Fig. 6) and those obtained by the application of a routine method based on total CHO assay kits in a diagnostic laboratory. As a final result, sesame oil had an acceptable role on decreasing CHO levels in rat's blood serum and this study suggests consumption of sesame oil in daily diet especially to those who suffer from high levels of CHO in their blood stream.

Figure 6

4. Conclusions

In this work, a novel and high performance amperometric CHO biosensor was fabricated based on co-immobilization of ChO, ChE and HRP onto Au NPs/Ch-IL/PEDOP/Gr-MWCNTs-Fr-IL/GCE by cross-linking enzymes and Ch through addition of glutaraldehyde with the aim of determination of CHO levels in blood serum samples. By the application of suitable modifications to the bare GCE, the biosensor exhibited fast response, good selectivity, high sensitivity and stability, and acceptable repeatability and reproducibility. Our records showed that the sensitivity of the biosensor was 6.6 $\mu A\ \mu M^{-1}\ cm^{-2}$, its response time was less than 5 s and the Michaelis-Menten constant was 0.12 μM. Comparing characteristics of the fabricated biosensor in this study with the other biosensors reported in the literature showed that the proposed this biosensor had better

results over the most of the reported biosensors so far. To verify the ability of the biosensor for the analysis of real samples towards CHO determination, it was applied to serum samples drawn from rats fed high CHO diet and sesame oil and fortunately, the biosensor was successful in the analysis of real samples and our records confirmed that the sesame oil had a great role on decreasing CHO levels in blood serums.

Acknowledgements

Hereby, I, Jalalvand AR, on the behalf of the authors express my sincere appreciation to Research Council of Kermanshah University of Medical Sciences to provide fund and time to do this project.

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Caption to figures:

Fig. 1. The corpses of the rats of (A) control group fed mouse pellets, (B) group 1 fed mouse pellets+10% (w/w) sesame oil, (C) group 2 fed a high cholesterol diet consisting of mouse pellets+20% (w/w) fat and (D) group 3 fed mouse pellets+20% (w/w) fat+10% (w/w) sesame oil after drawing blood samples by the cardiac puncture.

Fig. 2. The SEM images of (A) Gr-MWCNTs-Fr-IL/GCE, (B) PEDOP/Gr-MWCNTs-Fr-IL/GCE, (C) Ch-IL/PEDOP/Gr-MWCNTs-Fr-IL/GCE, (D) Au NPs/Ch-IL/PEDOP/Gr-MWCNTs-Fr-IL/GCE and (E) ChOChEHRP/Au NPs/Ch-IL/PEDOP/Gr-MWCNTs-Fr-IL/GCE.

Fig. 3. (A) Cyclic voltammograms of (a) bare GCE, (b) Gr-MWCNTs-IL/GCE, (c) Gr-MWCNTs-Fr-IL/GCE, (d) PEDOP/Gr-MWCNTs-Fr-IL/GCE, (e) Ch-IL/PEDOP/Gr-MWCNTs-Fr-IL/GCE and (f) Au NPs/Ch-IL/PEDOP/Gr-MWCNTs-Fr-IL/GCE in the PBS (0.05 M, pH 7) in the absence of H_2O_2 and (B) as (A) but in the presence of 0.5 mM H_2O_2. Inset of (B) shows the CVs of Au NPs/Ch-IL/PEDOP/Gr-MWCNTs-Fr-IL/GCE upon increasing concentration of H_2O_2: 0.5, 0.6, 0.8 and 1.5 mM.

Fig. 4. (A) Amperometric responses of ChOChEHRP/Au NPs/Ch-IL/PEDOP/Gr-MWCNTs-Fr-IL/GCE to increasing CHO concentrations (0.1-950 μM) upon applying a potential of -0.15 V and stirring the solution at 1000 rpm. (B) Calibration curve obtained by regression of current values on concentrations of CHO.

Fig. 5. (A) Amperometric response of the biosensor against injections of CHO (each injection contains 20 μM CHO), glucose (GL, 100 μM), ascorbic acid (AA, 200 μM), uric acid (UA, 150 μM), urea (UR, 250 μM) and bilirubin (BL, 250 μM) in the PBS (0.05 M, pH 7) containing 1% Triton X-100 (v/v) and 20% (v/v) isopropanol stirred at 1000 rpm and applying a potential of -0.15 V and (B) Long-term stability of amperometric response of the biosensor to CHO (100 μM) in the PBS (0.05 M, pH 7) containing 1% Triton X-100 (v/v) and 20% (v/v) isopropanol stirred by 1000 rpm and applying a potential of -0.15 V during six weeks.

Fig. 6. Amperograms recorded by the application of the proposed biosensor to the analysis of blood serums of the rats belong to (A) control group, (B) group 1, (C) group 2 and (D) group 3 towards CHO determination upon applying a potential of -0.15 V and stirring the solution by 1000 rpm. a→e refers to the amperograms recorded from the analysis of serum samples of the rats 1-5 in each group. Each sample was diluted 1000 times and analyzed upon three injections.

Scheme 1. Schematic representation of the steps of this study.

Highlights

- ✓ EDOP was electropolymerized onto Gr-MWCNTs-Fr-IL/GCE.
- ✓ Ch-IL was drop casted onto PEDOP/Gr-MWCNTs-Fr-IL/GCE.
- ✓ Au NPs were electrochemically deposited onto Ch-IL/PEDOP/Gr-MWCNTs-Fr-IL/GCE.
- ✓ ChOChEHRP were co-immobilized onto the sensor surface.
- ✓ Biosensor was used to verify the effects of sesame oil on CHO levels in rat plasma.

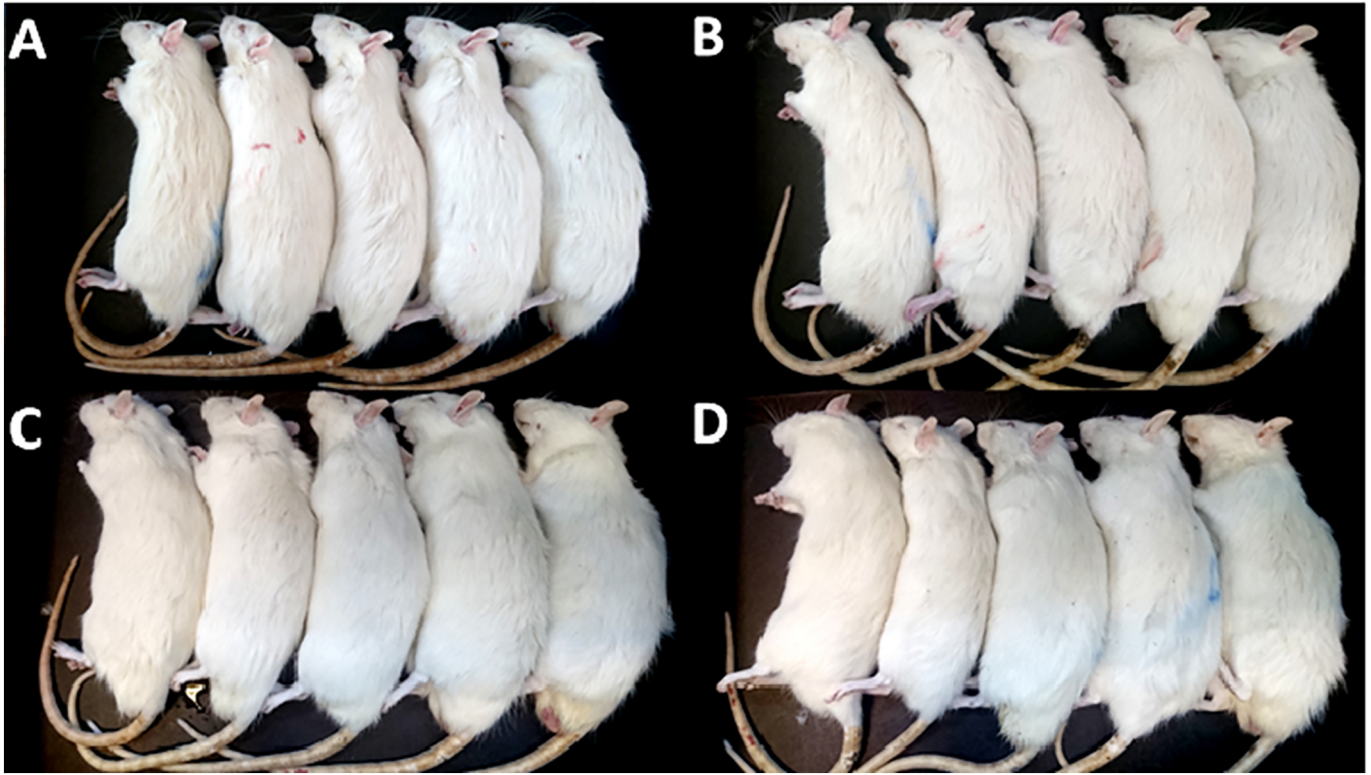
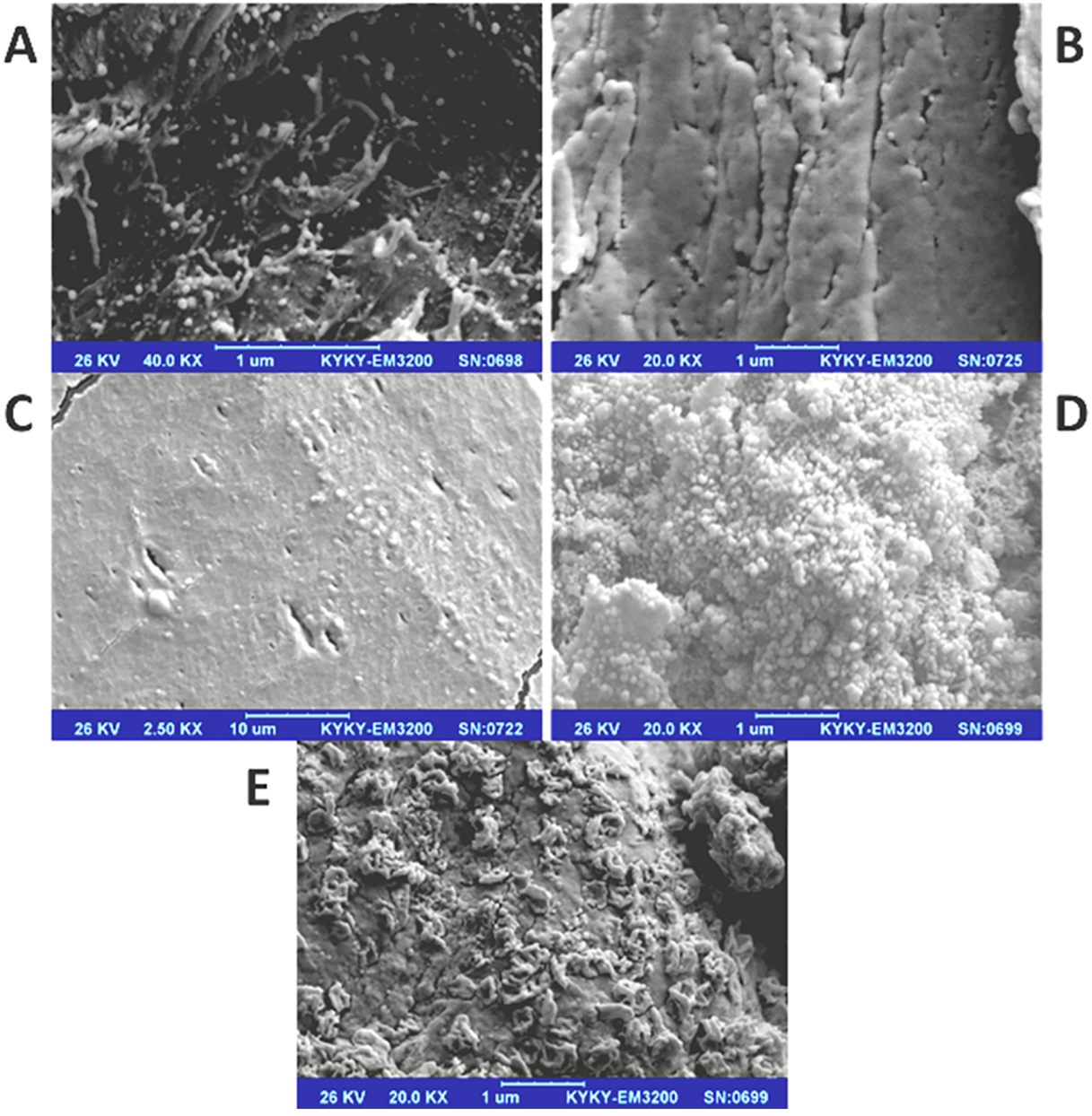


Figure 1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B
26 KV 20.0 KX 1 um KYKY-EM3200 SN:0725
C
26 KV 2.50 KX 10 um KYKY-EM3200 SN:0722
D
26 KV 20.0 KX 1 um KYKY-EM3200 SN:0699
E
26 KV 20.0 KX 1 um KYKY-EM3200 SN:0699

Figure 2

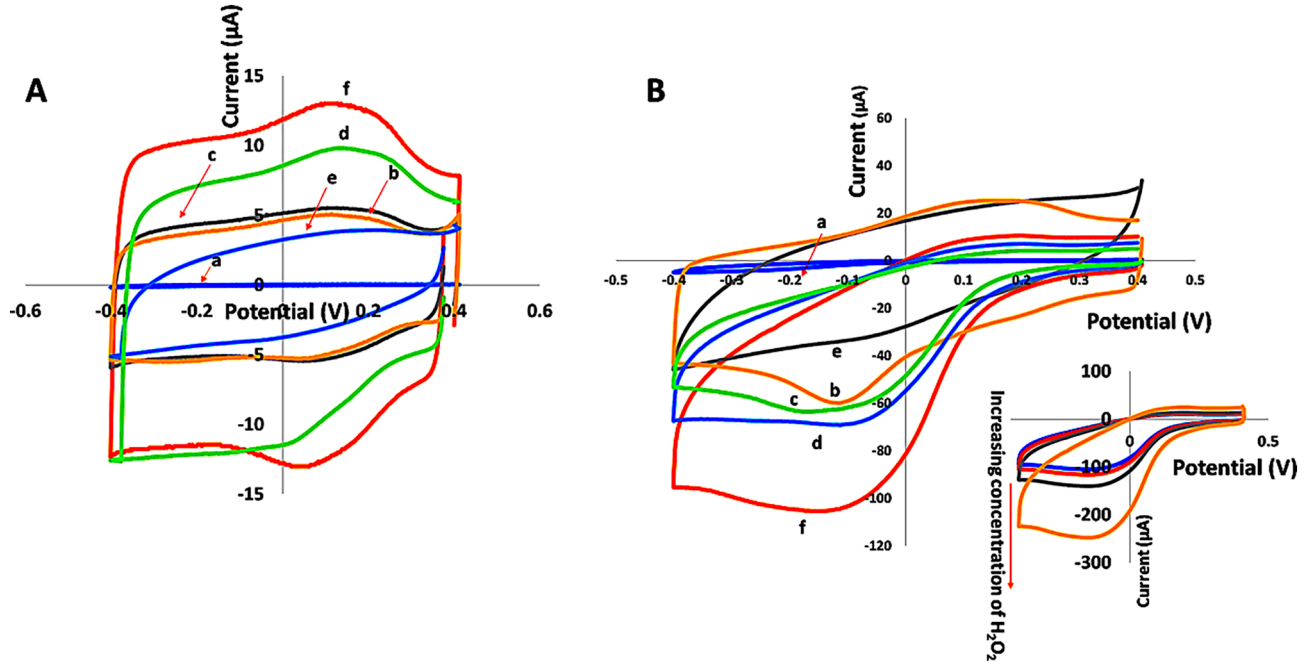

A
Current (μA)
Potential (V)
a
b
c
d
e
f
B
Current (μA)
Potential (V)
Increasing concentration of H_2O_2
Current (μA)
Potential (V)

Figure 3

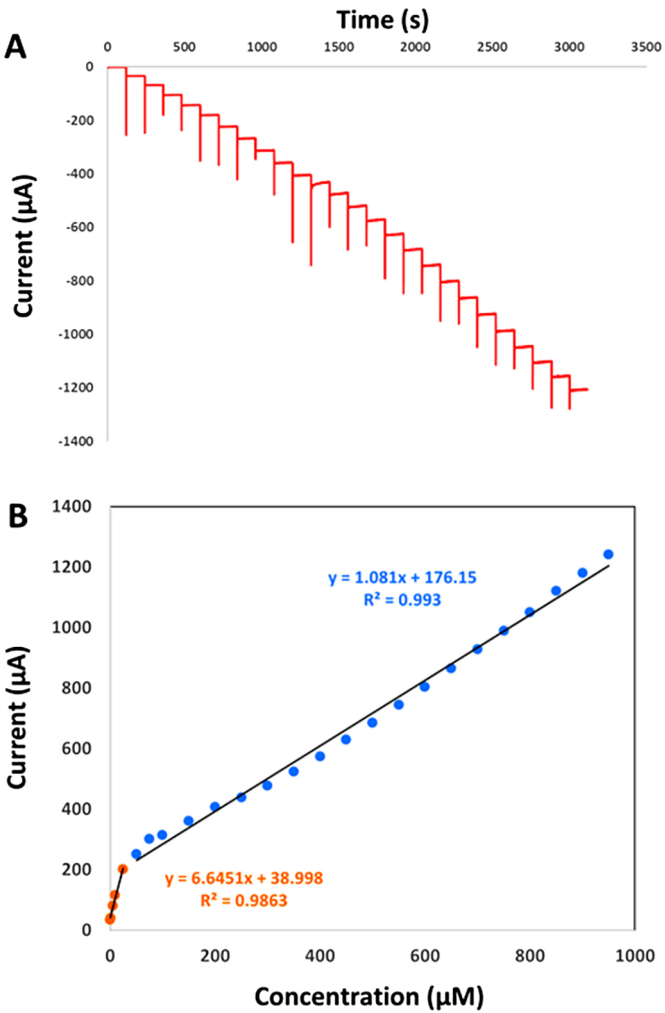
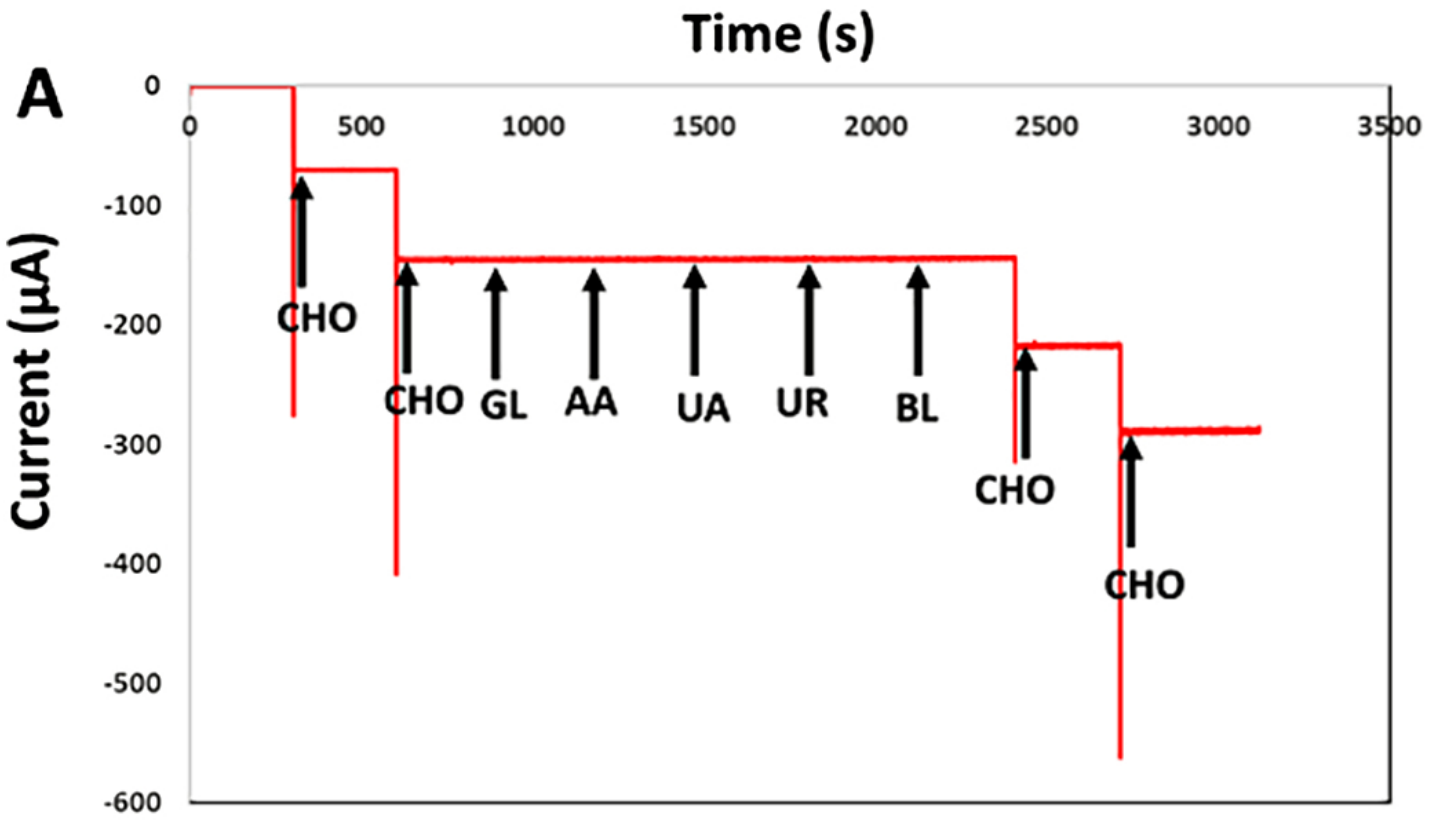

A
Time (s)
0
500
1000
1500
2000
2500
3000
3500
Current (µA)
0
-200
-400
-600
-800
-1000
-1200
-1400
B
Current (µA)
1400
1200
1000
800
600
400
200
0
y = 1.081x + 176.15
R² = 0.993
y = 6.6451x + 38.998
R² = 0.9863
0
200
400
600
800
1000
Concentration (µM)

Figure 4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Current (μA)
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CHO
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GL
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UA
UR
BL
CHO
CHO

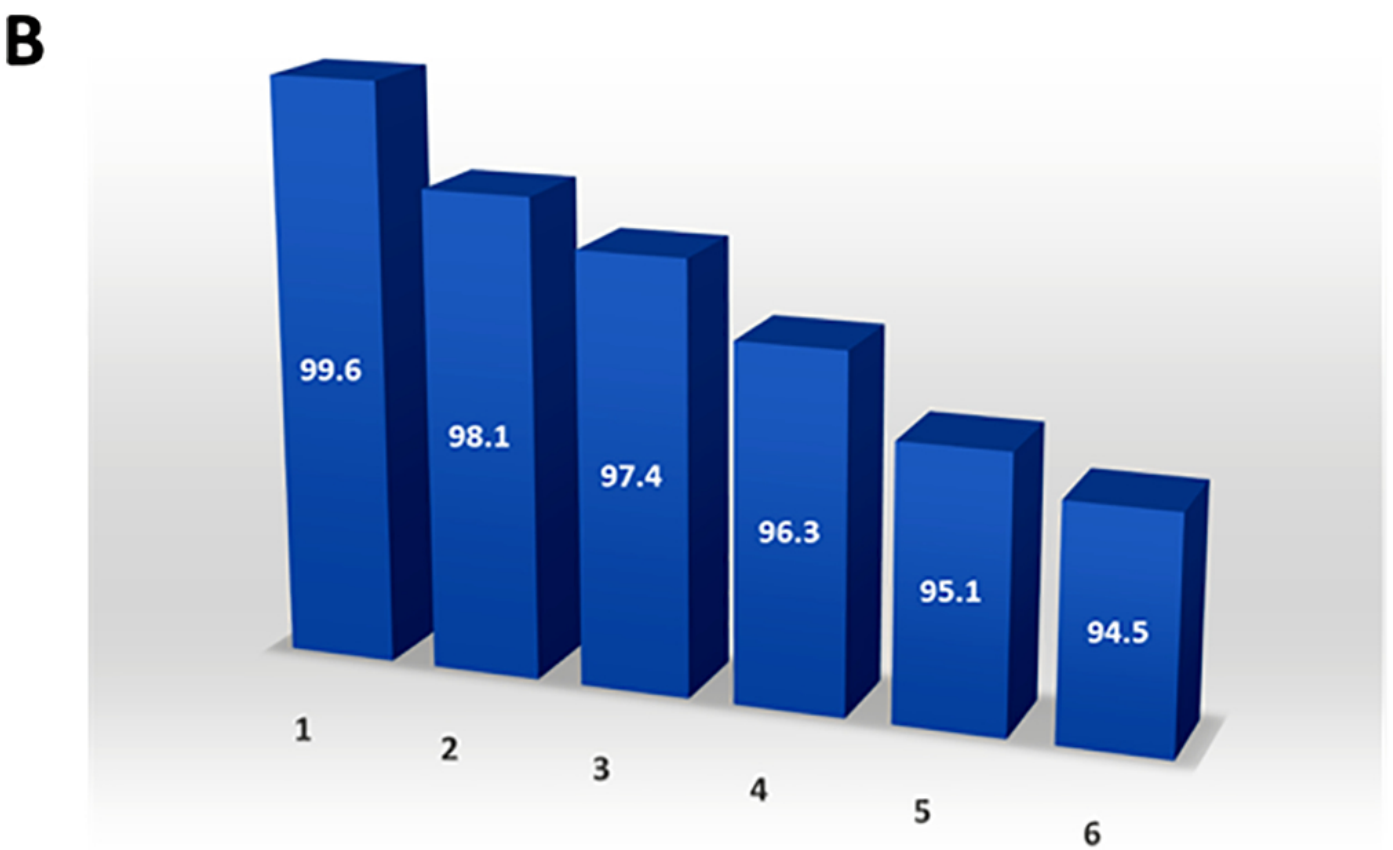
B
99.6
98.1
97.4
96.3
95.1
94.5
1
2
3
4
5
6

Figure 5

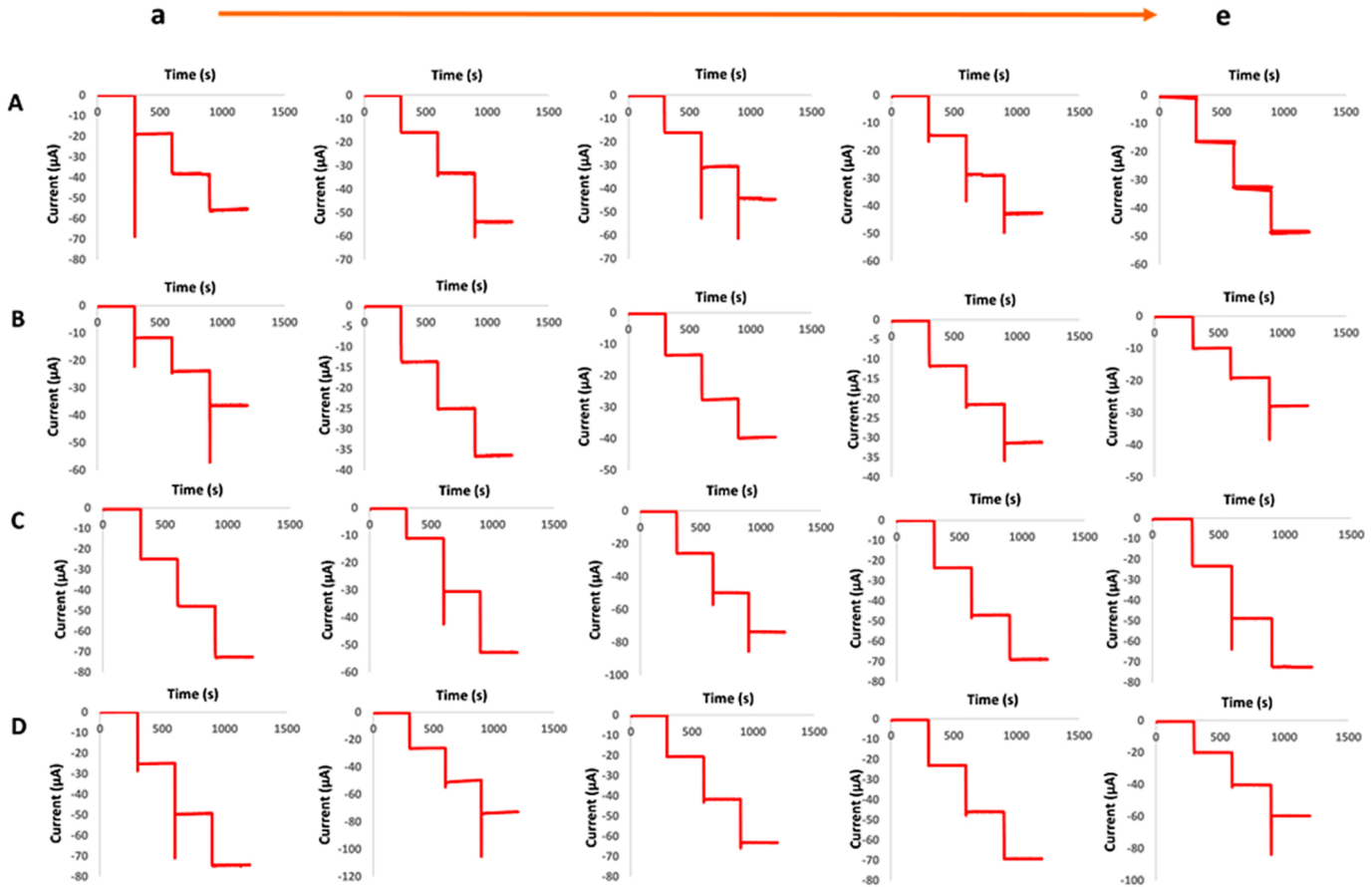

Figure 6