

Direct electrochemistry of hemoglobin in a renewable mesoporous carbon ceramic electrode: a new kind of hydrogen peroxide biosensor

Biuck Habibi · Mojtaba Jahanbakhshi

Received: 30 June 2014 / Accepted: 5 November 2014 / Published online: 22 November 2014
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Abstract A sol–gel derived renewable biosensor was prepared by entrapping hemoglobin (Hb) in a mesoporous carbon ceramic electrode (MCCE) in order to study the direct electron transfer of Hb and to fabricate a sensitive biosensor for hydrogen peroxide (H_2O_2). The Hb in the MCCE retained its original structure and still displayed good electrochemical activity in showing a pair of well-defined redox peaks as a result of direct electron transfer between Hb and the electrode surface. The MCCE possesses good biocompatibility, renewability, uniformity, and is well suited for immobilization of enzymes. The immobilized Hb was found to display good electrocatalytic activity for the reduction of H_2O_2 , and this resulted in the design of an amperometric biosensor working at -0.3 V and capable of sensing H_2O_2 with a detection limit of $0.4 \mu M$ (at a S/N of 3). The catalytic reduction current is proportional to the concentration of H_2O_2 in the 1.0 to $220.0 \mu M$ range. This work demonstrates MCCE to be a well suited and easily accessible substrate for engineering biosensors with various kinds of potential applications.

Keywords Hemoglobin · Hydrogen peroxide · Biosensor · Mesoporous carbon · Sol–gel

Introduction

Nowadays amperometric biosensors, due to their good selectivity and high sensitivity, have attracted much attention; meanwhile, the improvement of the assistant matrix for the

immobilization of the enzymes or proteins is the basic purpose of the amperometric biosensing researches. Unfortunately, the designed biosensors show lower stability and durability because of aloofness of the proteins and enzymes or conformational change; therefore, finding an appropriate matrix for immobilization of redox centers is important for obtaining their direct electrochemical reaction and keeping their bioactivities [1]. In this context, immobilization of proteins and enzymes on/in the three-dimensional matrices is a promising direction which results in rapid advances in biosensing researches. Recently, various three-dimensional matrices were tested for immobilization of proteins or enzymes and then were used for the fabrication of electrochemical biosensors [2, 3]. On the other hand, mesoporous materials have become a common choice when selecting immobilization support due to some unique advantages such as high specific surface area, well-ordered pore structure, narrow pore size distribution, mechanical and chemical resistance [4–6]. Therefore, they are promising materials for the loading of biomolecules. Among them mesoporous carbon (MC), due to a large number of mesopores, well-defined pore size, flexible framework composition and good biocompatibility, attracted much attention [7, 8]. Also, contrary to most porous silica-based materials such as SBA-15 [9] and zeolite [5] that are electronic semiconductors, MC is intrinsic conductor. Hence, MC may have more interests and potential advantages for many advanced applications than other porous materials [10–12]. Despite such potential capability of MC, there are a few reports on the immobilization of biomolecules such as hemoglobin (Hb) on/in the MC [13] and its application as an enzymatic sensor for H_2O_2 determination.

Hb, a tetrameric protein that consists of four polypeptide chains, contains a heme group which acts as the active center to store and carry oxygen in the blood cells and is a perfect component for the investigation of electron transfer performance of proteins and correspondingly for the development of

Electronic supplementary material The online version of this article (doi:10.1007/s00604-014-1403-6) contains supplementary material, which is available to authorized users.

B. Habibi (✉) · M. Jahanbakhshi
Electroanalytical Chemistry Laboratory, Department of Chemistry,
Faculty of Sciences, Azarbaijan Shahid Madani University,
Tabriz 53714-161, Iran
e-mail: b.habibi@azaruniv.edu

biosensors [5, 9]. Electrocatalytic activity of Hb toward hydrogen peroxide (H_2O_2) reduction was demonstrated in the previous reported literatures [2, 3, 5, 9, 13–16]. To our best knowledge, there isn't any report about the direct electrochemistry of heme protein immobilized in mesoporous carbon ceramic electrode (MCCE) as a renewable modified electrode and utilized to construct Hb entrapped mesoporous carbon ceramic electrode (Hb/MCCE) biosensor.

In this work, we entrapped the Hb into the MC sol–gel matrices as a novel and renewable MC ceramic electrode: Hb/MCCE. The constructed biosensor displayed fast electron transfer and prominent catalytic ability toward H_2O_2 reduction with wide linear range and low detection limit. On the other hand, because of good stability, ease of fabrication, renewability and special catalytic properties, MCCE can be used as an artificial medium which suggests that this substrate has an extensive range of potential uses in biosensor developing. These arresting properties of the MC combined with the benefits of sol–gel process mainly propose that the sol–gel derived MC materials are much more valuable for electrochemical studies than the conventional carbon ceramic electrode (CCE) (which is in the same family). Therefore, this work opens a new way to broaden the applications of MCCE in electrochemical biosensors.

Experimental

Chemicals and apparatus

Hb was purchased from sigma chemical Co. (USA) and MC was prepared via silica mesoporous as a template and glucose as carbon source. All of the other chemicals were of analytical grade and were used without additional purification. All of the solutions were prepared with distilled water. Pure nitrogen gas (N_2) was applied for the elimination of oxygen from solutions to prevent the conceivable oxygen reaction throughout the experiments.

AUTOLAB PGSTAT-100 (potentiostat/galvanostat) (www.metrohm-autolab.com) is equipped with a three-electrode system, Hb/MCCE, a platinum wire electrode and a silver/silver chloride (satd. KCl) electrode (Ag/AgCl) (Azar Electrode Co., Iran) respectively as working, counter and reference electrodes, and was used to complete the electrochemical experiments. The morphological and structural characterizations of the Hb/MCCE were specified by field emission scanning electron microscopy (FE-SEM) on a JSM-6700 F (JEOL Ltd., Japan) (www.jeolusa.com/) device and the JEM-1200 EX/S transmission electron microscopy (TEM).

Preparation of Hb/MCCE

Mesoporous silica was fabricated by the described process [17]. The fabricated mesoporous silica was used as the MC template in the synthesizing procedure (Electronic Supplementary Material, ESM). For the preparation of Hb/MCCE, first 50 mg of Hb was dissolved in the 5.0 mL cold water (4 °C) and then it was mixed with 200 mg of MC at 4 °C while it was stirring for about 5 hours. The combination was left to dry in desiccator at 4 °C for about 48 h. Silica sol was prepared according to the following process: 0.30 mL of methanol, 0.15 mL of methyltrimethoxysilane and 10 μ L of HCl (11 M) were mixed. This mixture was sonicated about 0.5 h and stored at room temperature for 24 h, after which Hb-impregnated MC was added and the obtained combination was shaken for 1 min at 4 °C. A 5-mm length of a Teflon tube (3–4 mm i.d.) was filled with the sol–gel Hb/MC mixture and dried for a week at 4 °C. The CCE and Hb/CCE were also prepared in the same way as above, except that the carbon powder was used instead of MC.

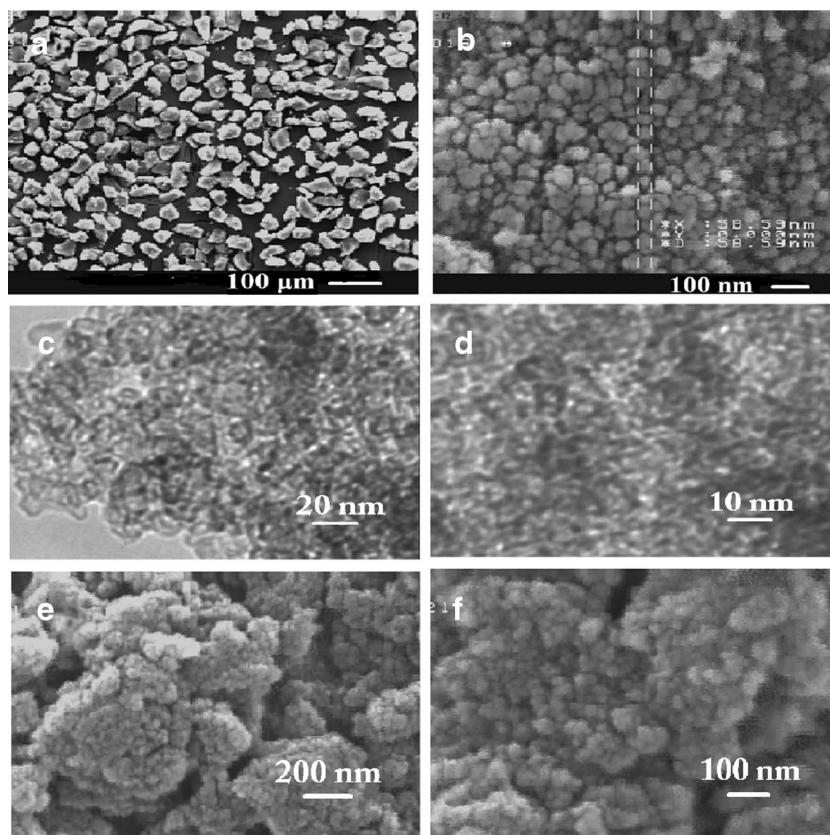
Results and discussion

Characteristics of MC and MCCE surface

The characteristic FE-SEM images of the prepared MC with low (Fig. 1a) and high magnification (Fig. 1b) are shown in Fig. 1. Figure 1a demonstrates the uniform structure with approximately equal sizes for the fabricated MC particles. The high magnification displays that the MC consists of small spherical units with the particle sizes ranging from 20 to 50 nm (Fig. 1b). Furthermore the examination was accomplished by TEM. Figure 1 also shows the TEM images of synthesized MC in this work (Fig. 1c and d). These images show the separate pores with three dimensional unified channel organizations. The fabricated MC reveals a pore diameter of about 2–5 nm with the wall thickness of around 5 nm. The obtained results show that the resulted MC can be applied as a suitable matrix for enmeshment of biological compounds in biosensor development.

Moreover, the surface images of fabricated MCCE with low (Fig. 1e) and high (Fig. 1f) magnification are also shown in Fig. 1. The morphology of MCCE surface is gritty with a three-dimensional and porous arrangement. This proves that MC materials are surrounded by the jagged network of the sol–gel prepared electrode and formed a porous medium which creates a fitting matrix for capturing hemoproteins or enzymes.

Fig. 1 FE-SEM images of the MC with low (a) and high magnification (b). TEM images of MC with different, low (c) and higher (d), magnification. Low (e) and high (f) magnification FE-SEM images of the MCCE surface



Direct electrochemistry of Hb/MCCE

The direct electrochemistry of Hb in the fabricated Hb/MCCE was studied by cyclic voltammetry. Curve a in Fig. 2 illustrates the cyclic voltammogram of the Hb/MCCE in 0.1 M, pH 7.0, N₂-saturated phosphate buffer solution at the scan rate of 100 mV/s. The Hb/MCCE showed a couple of well-defined and quasi-reversible redox peaks at -0.236 V (E_{pc}) and -0.085 V (E_{pa}) with a peak-to-peak separation of about 150 mV. This represents the characteristic of heme Fe(III)/Fe(II) redox couples of Hb [3, 6, 18, 19] which indicated that the direct electron transfer of Hb with MCCE had taken place and MCCE can realize the direct electrochemistry of Hb. On the other hand, in order to compare the electron transfer of Hb in the MCCE with CCE (which is in the same family), direct electrochemistry of Hb trapped into CCE (Hb/CCE) was explored using cyclic voltammetry in N₂-saturated 0.1 M phosphate buffer solution (pH 7.0). According to Fig. 2 curve b, no redox peak was perceived for the Hb/CCE. This phenomenon reveals that direct electron transfer between Hb and MCCE was greatly enhanced and MCCE could provide a favorable 3D material for the proteins and could play an important role in improving the electron exchange, due to its good biocompatibility, large surface, large number of mesopores, and high conductivity. In other words, it can be concluded that the MCCE has an immense effect on the kinetics of electrode reaction and produces a satisfactory

environment for the Hb to transfer electron in the prepared electrode and provides a favorable nanoenvironment to avoid denaturation of Hb molecules [20, 21]. Additionally, as it can be seen in Fig. 2 curve a, the shape of the anodic to cathodic peaks are approximately symmetric, and the reduction and oxidation peak heights are similar. These behaviors suggest that all of the electroactive HbFe(III) centers on the electrode surface are transformed to HbFe(II) in the sweep forward to negative potential and vice versa.

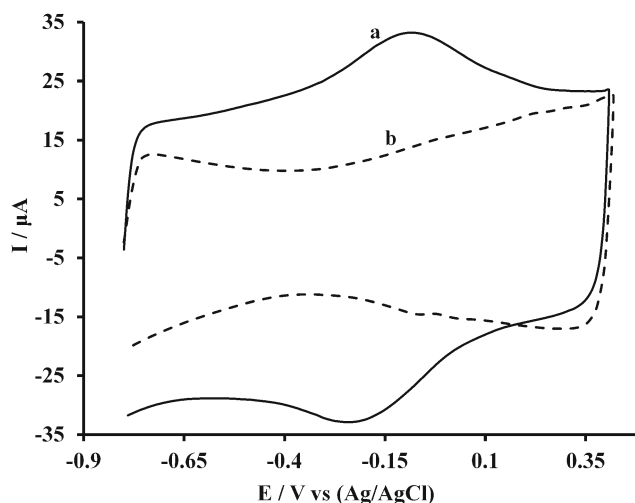


Fig. 2 Cyclic voltammograms of Hb/MCCE (a) and Hb/CCE (b), in 0.1 M phosphate buffer solution (pH 7.0), scan rate: 100 mV s⁻¹

To further investigate the characteristics of Hb at the Hb/MCCE electrode, the effect of the varying scan rate on the peak current was investigated. The cyclic voltammograms of Hb/MCCE at various scan rates were shown in Fig. 3. The peak current and also the separation of peak to peak increase with the increasing scan rate. The anodic and cathodic currents (I_{pa} and I_{pc}) of the immobilized Hb are linearly proportional to the scan rate ($I_{pa}=0.4941v - 11.896$, $R^2=9968$ and $I_{pc} = 0.4847v + 9.799$, $R^2=9976$, respectively) in the range of 20 to 300 mVs^{-1} and this indicates a characteristic surface-controlled electrode procedure [5, 22]. According to Laviron's equations [23], the potentials of the anodic and cathodic peaks were linearly reliant on the scan rates (v) logarithm with the slopes of $2.3RT/(1-\alpha)nF$ and $-2.3RT/\alpha nF$, correspondingly (Fig. S1, ESM). The value of the electron transfer coefficient (α) was estimated to be 0.57 and the rate constant (k_s) of heterogeneous electron transference was calculated with the following equation [23]:

$$\log k_s = \alpha \log (1-\alpha) + (1-\alpha) \log \alpha - \log RT/nFv - \alpha (1-\alpha) nF \Delta E_p/2.3RT \quad (1)$$

where α is the coefficient of charge transfer, v is the scan rate, ΔE_p is the separation of anodic to cathodic peak potential, n is the electron transfer number and the F , R and T symbols have their ordinary meanings. From the Eq. (1), k_s was estimated to be $1.05 s^{-1}$. This value is much larger than those reported for the Hb immobilized on the other materials: AuNPs ($0.490 s^{-1}$) [24], Hb/C₆₀-MWCNT ($0.39 s^{-1}$) [25], CNT powder ($0.0620 s^{-1}$) [26], macroporous gold ($0.950 s^{-1}$) [27],

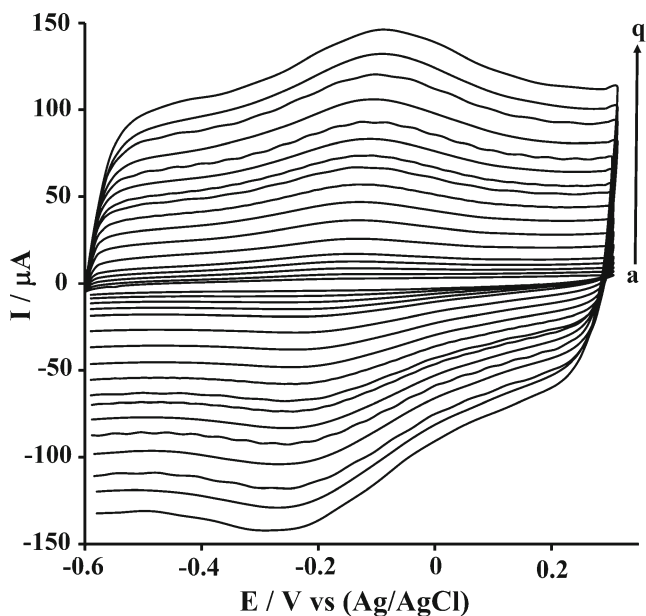


Fig. 3 Cyclic voltammograms of Hb/MCCE in 0.1 M phosphate buffer solution (pH 7.0) at various scan rate 20, 30, 40, 50, 60, 70, 80, 100, 120, 140, 160, 180, 200, 220, 240, 260 280 and 300 $mV s^{-1}$ (a-q)

$Fe_3O_4@AuNPs$ ($1.03 s^{-1}$) [22], PSMAC/MWCNTs ($0.84 s^{-1}$) [28], nano $CaCO_3$ ($0.75 s^{-1}$) [29], SnO_2 ($0.53 s^{-1}$) [30], TiO_2 ($0.137 s^{-1}$) [31], and WO_3 ($0.97 s^{-1}$) [32]. Such results indicate that the MCCE provides a suitable nanoenvironment for increasing the electron transfer rate of Hb.

Finally, the effect of pH on the E_p of anodic and cathodic waves was examined and a slope of about $-60 mV/pH$ was obtained for both of them (Fig. S2, ESM).

Electrocatalytic reduction of H_2O_2 at the Hb/MCCE

Proteins and enzymes with heme group such as Hb, cytochrome c, horseradish peroxidase and myoglobin have high ability in reducing H_2O_2 electrocatalytically [2, 3, 5, 13]. So to explore the activity of Hb at the Hb/MCCE, its performance to the H_2O_2 reduction was deliberated. Fig. 4 exhibits the cyclic voltammograms of the Hb/MCCE in 0.1 M phosphate buffer solution (pH 7.0) and in the absence (a) and presence (b-e) of H_2O_2 . The comparison of curve a and curves b to e reveals that after H_2O_2 addition, the reduction peak current growth goes along with decrease in the oxidation peak current. These behaviors show that the catalytic H_2O_2 reduction really develops from the Hb captured on/in the MC-silica network. The obtained results show that the immobilized Hb in the MC matrix reserved its bioactivity on/in the electrode and exhibited admirable electrocatalytic performance toward the H_2O_2 reduction. The common reaction for H_2O_2 reduction by Hb can be stated as following [26]:

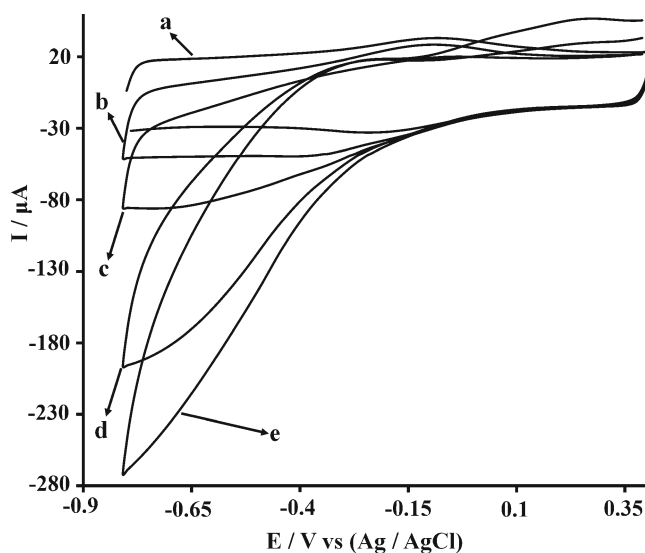
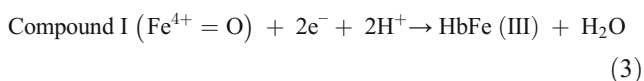


Fig. 4 Cyclic voltammetry response of Hb/MCCE in the presence of 0 (a), 0.5 (b), 1 (c), 5 (d) and 10 mM of H_2O_2 in 0.1 M phosphate buffer solution (pH 7.0), scan: rate 100 $mV s^{-1}$

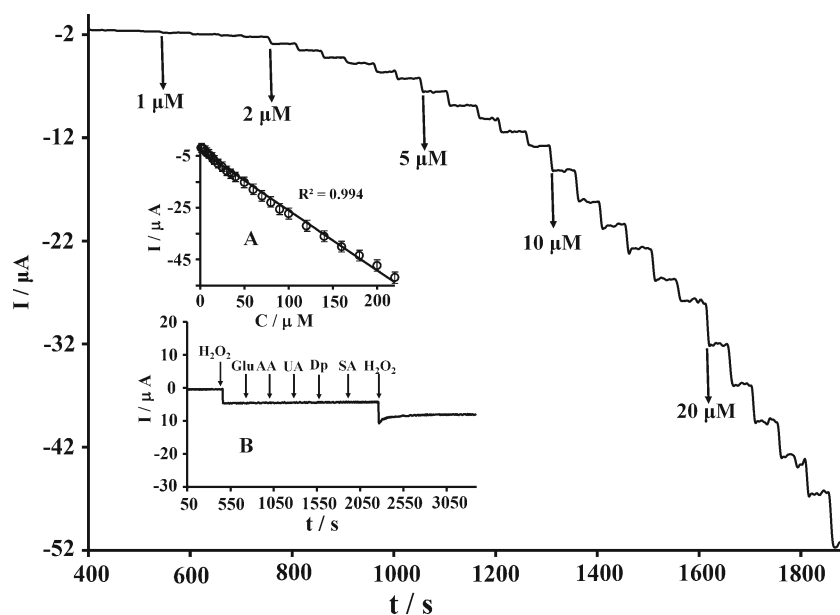


Corresponding to the above equations, the added H₂O₂ upsurges the heme group oxidation form in the vicinity of the modified electrode, causing to amplifying the reduction current and vanishing the anodic current. On the other hand, as it might be expected, insignificant response to H₂O₂ was originated for the Hb/CCE (data not shown here).

Amperometric detection of H₂O₂ at the Hb/MCCE

The capability of the obtained biosensor was examined by challenging the Hb/MCCE with H₂O₂ increasing concentrations. The amperometric trial was performed in a stirred cell containing 0.1 M phosphate buffer solution (pH 7.0) in the optimized working electrode potential of −0.3 V. Figure 5 shows the cathodic current responses of the Hb/MCCE to frequent additions of H₂O₂ concentrations. The reaction happening at the Hb/MCCE very rapidly reaches a dynamic steadiness upon each addition of the H₂O₂, producing a steady-state current signal within 5–10 s. As exhibited in inset A of Fig. 5, the obtained linear range is around 1.0 to 220.0 μM (R²=0.994), and the detection limit is estimated to be 0.4 μM (S/N=3). The apparent Michaelis-Menten constant (K_M) which gives an indication of the enzyme-substrate kinetics and provides a way to compare the concerned H₂O₂ sensor with others can be calculated from the Lineweaver-Burk equation [34] (1/I_{SS}=K_M/I_{max}C+1/I_{max}), where I_{SS} is the steady-state current after the addition of substrate, C is the bulk concentration of the H₂O₂, and I_{max} is the maximum current measured under saturated substrate conditions. The K_M for the Hb/MCCE was estimated to be 0.125 mM

Fig. 5 Amperometric responses of Hb/MCCE to stepwise addition of H₂O₂ (1.0 to 220 μM) in 0.1 M phosphate buffer solution (pH 7.0) under the conditions of −0.30 V constant potential and rotation speed is 1000 rpm. Plot of amperometric current vs. H₂O₂ concentration, inset (a). Current-time curves for the Hb/MCCE exposed to 0.02 mM H₂O₂ and Glu, AA, UA, Dp, SA, 0.1 mM each compound and finally 0.02 mM H₂O₂, inset (b)



(Fig. S3, ESM), which is smaller than those of Hb in sodium alginate-MWCNTs composite film of 0.533 mM [34] and Hb in silica sol-gel film of 0.898 mM [35]. This indicates that Hb entrapped in the Hb/MCCE exhibits a high affinity and catalytic activity to H₂O₂. Moreover with the purpose to demonstrate the applicability of the obtained biosensor for real sample analysis, 10, 50, 100 and 200 μM H₂O₂ were added into rain water samples, respectively. The average recovery of the biosensor was 98.1 %, 96.3 %, 101.5 % and 102.0 % (n=5), respectively. The mean value of average recovery, 99.48 %, indicates that the potential practical application of Hb/MCCE biosensor for H₂O₂ in real samples.

Finally the response of the resultant Hb/MCCE to the usual interfering species such as salicylic acid (SA), glucose (Glu), ascorbic acid (AA), uric acid (UA), dopamine (Dp) and salicylic acid (SA) was similarly estimated under the optimized condition (−0.3 V and pH 7.0). This perfect biosensor showed no response to 0.1 mM of each of these compounds (inset B of Fig. 5). The capability of the fabricated biosensor applied in this work was compared with some other reported immobilized Hb biosensors (Table S1, ESM). This comparison discloses that our planned biosensor exhibits efficient performance for H₂O₂ determination.

The Hb/MCCE stability, repeatability, reproducibility and renewability

The stability of the biosensor was investigated over about 2 weeks with the storing of Hb/MCCE in 0.1 M phosphate buffer solution (pH 7.0) at 4 °C. The biosensor reserved 95 % of its initial activity after 2 weeks. The biosensor repeatability and reproducibility were estimated [36, 37] in the presence of 5.0 μM of H₂O₂ in 0.1 M phosphate buffer solution (pH 7.0)

via 5 repetitive tests and 5 separate modified electrodes were prepared in the same way for amperometric detection of H_2O_2 in the optimal operating conditions. The relative standard deviation (RSD) was 2.4 % for 5 consecutive evaluations and 3.5 % for 5 separate biosensors, respectively. In order to study the renewability of the individual Hb/MCCE, its responses were verified in the presence of 5.0 μM of H_2O_2 in 0.1 M phosphate buffer solution (pH 7.0) after each polish. The R.S.D. value of the measured anodic peak currents for five-time polish and use was about 3.8 %, which reveals very good polishing procedure. Therefore, the Hb/MCCE was stable in the storing condition just as the stability of the conventional graphite or glassy carbon electrodes, but its surface could be refreshed by polishing. These results show that the Hb/MCCE is a stable, repeatable, reproducible and renewable modified electrode which can be used in the H_2O_2 biosensing.

Conclusion

This study has effectively established a capable H_2O_2 biosensor based on Hb entrapped in the MCCE via a simple and effective immobilization method. Direct electrochemistry investigation of Hb/MCCE demonstrated high electron transfer for Hb stabilized on/in the MCCE. The prepared Hb/MCCE biosensor successfully sensed H_2O_2 with short response time, long linear range and low limit of detection. The obtained biosensor indicates actual high sensitivity, long-term stability, excellent repeatability, reproducibility and renewability. The present biosensor could detect H_2O_2 in a linear range of 1.0 to 220.0 μM and the detection limit was found around 0.4 μM (S/N=3). The optimistic consequences obtained in this research demonstrate that the MC-silica network is an admirable matrix for protein and enzyme entrapment, introducing a new way for the progression of high-performance electrochemical biosensors. On the other hand, the main advantage of this biosensor is its facile and fast preparation in one polishing stage. In sum, the biosensor planned in this research is very promising in the recognition of H_2O_2 because of the advantages of low cost, easy preparation, high sensitivity, fast response and renewability.

Acknowledgment The authors are grateful to the Azarbaijan Shahid Madani University Research Office (Grant No. 214/d/10802), Tabriz, Iran for financial support.

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