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ORIGINAL PAPER

Myoglobin immobilized on mesoporous carbon foam in a hydrogel (selep) dispersant for voltammetric sensing of hydrogen peroxide

Mojtaba Jahanbakhshi [1]

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Abstract

Mesoporous carbon foam (MCF) was prepared by via the Pechini method which is facile and template-free. The MCF was characterized by field emission scanning electron microscopy, transmission electron microscopy, X-ray diffraction and BET surface area analysis. Afterwards, the MCF was dispersed in the natural hydrogel salep to give a composite. Finally, myoglobin was immobilized on the composite and then placed on a glassy carbon electrode (GCE). The modified GCE gives a distinct quasi-reversible redox peak during electroreduction of hydrogen perxide (H_2O_2). The estimated electron transfer coefficient (α) and the heterogeneous electron transfer rate constant (ks) for redox process of Mb are 0.54 and 2.25 s^{-1}, respectively. The sensor, best operated at −0.2 V (vs. Ag/AgCl), responds to H_2O_2 in the 1.0 to 80 μM H_2O_2 concentration range, with a 180 nM limit of detection (at S/N ratio of 3). The technique was applied to the determination of H_2O_2 in spiked fetal bovine serum samples.

Keywords Pechini method · Mesoporous carbon foam · Salep flour · Myoglobin · Electroanalysis

Introduction

The direct electron transfer between proteins and electrode surface has been widely considered since the first reports about immobilization of cytochrome c on tin-doped indium oxide electrodes [1]. Myoglobin (Mb is an essential oxygen-transporter in mammalian skeleton and muscle structures [2]. Mb has been applied as an ideal model system for research on direct electron-transfer (DET) of hemoproteins and enzymes owning to its commercial availability, well- known structure and enzyme-like performance [2, 3]. It is worthwhile to select a substrate on which these proteins ca be immobilized and as a result, the DET between these molecules and electrode surface is facilitated.

Carbon based nanocomposites have been applied as a substrate for the immobilization of hemoproteins on the electrode surface [4, 5]. Among them, mesoporous carbon (MC) has high stability and efficient electronic features. MC possess numerous exclusive characteristics, such as meso-structure, abundant pores, admirable mass transfer, distinct pore size, excessive specific surface area, low dielectric constant and great pore volume [4, 6]. Therefore, MC has been applied as a suitable substrate for Mb immobilization and final application for electrode modification. The electrochemical properties of MC is well studied. The features and capabilities of mesoporous materials, e.g. MC, in electrochemical devices (sensors and biosensors) have been explained in detail in previously published review [7]. Also, large number of procedures for MC fabrication [8–10], and its application in sensors and biosensors have been reported [11–13].

The use of MC, especially in electrochemical devices, suffers from two main drawbacks. First, MC synthesis requires a special template and second, defect is the low dispersity of pristine MC in aqueous medium. In order to tackle these unresolved issues, there is a need for simple synthesis method of MC with low cost and template-free features. Moreover, an appropriate medium with high ability to disperse the pristine MC without covalent functionalization is required since the covalent functionalization results in broking the MC structure. Subsequently depletes the applicable properties of this material, is really desirable.

Pechini method is introduced to synthesize MC to enhance compositional homogeneousness [14, 15]. This method has been extensively applied in the nanocrystals synthesis owning

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✉ Mojtaba Jahanbakhshi
Moji.j2137@gmail.com

[1] Young Researchers and Elite Club, Shahrekord Branch, Islamic Azad University, Shahrekord, Iran

its low cost, non-toxicity, simplicity and template-free characteristics features compared to other methods [15]. Pechini method is carried out by a rigorous mixing of positive ions with a solution, organized conversion of the blend into a polymeric compound, and finally elimination of the polymer background and progress of a homogenous oxide precursor.

In order to synthesize mesoporous carbon (MC) using the Pechini method, polymeric compound is required to prepare the stable suspension. Hydrophilic polymers such as sodium alginate and chitosan are frequently used for MC synthesis due to theirs high ability as dispersant to create stable carbon materials suspensions [16, 17]. We introduced Salep for the first time as a dispersant for epinephrine detection previously [18]. Salep is a hydrophilic polymer, similar to sodium alginate and chitosan in structure and properties, with advantageous properties such as cheapness, high availability, non-toxicity and gelling possessions [19]. Salep is preferred over chitosan and sodium alginate due to its higher availability and lower price. In the previous work we introduced MCF as a new modifier, but in this work MCF was used as a novel substrate for Mb immobilization [18]. This work show that MCF can act as a suitable substrate for stabilizing myoglobin and facilitating its electron transport. A biosensor was developed in this work, but in the previous work, an electrochemical sensor was established. It is important that a substrate be able to immobilize a biomolecule, this can be a new platform for improving biosensors.

MC foam (MCF) with spread surface area and appropriate distribution of pore size is prepared here via template-free Pechini method. The method is improved compare to traditional Pechin method as a matter of some basically changes especially in metal precursor and final stage. The metallic production is removed and instead of metallic production, polymeric and chelating agents were used in final step of pechini method. As a result, the MCF is mixed with Salep and a composite of S-MCF is fabricated with admirable dispersion. Finally, Mb was immobilized on or into S-MCF composite and resultant hybrid was casted on the GCE surface to design a novel biosensor (S-MCF/Mb/GCE). The biosensor displayed a fast DET, and also noticeable catalytic activity toward H_2O_2 reduction with extensive linear range and low detection limit.

Materials and methods

Chemicals

Salep was purchased from local source (ShahreKord, Iran). All other reagents were in analytical grade and were purchased from Merck (http://www.merck.com) or Fluka (http://www.sigmaaldrich.com) and applied without further purification. The phosphate buffer (0.1 M, pH 7.0) was used as supporting electrolyte and doubly distilled water was used during the all of investigations.

Apparatus and measurements

All electrochemical analysis were performed by means of an potentiostat/galvanostat (AUTOLAB PGSTAT-100, metrohm-autolab, Switzerland) with a typical three-electrode association composed of an Ag/AgCl (3 M KCl) reference electrode, a platinum auxiliary electrode, and Mb/S-MCF/GCE as working electrode. The morphology of MCF were investigated through trans-mission electron microscopy (TEM) tool (JEM-1200 EX/S) and field emission-scanning electron microscopy (FE-SEM) device (JSM-6700F FE-SEM, JEOL Ltd., Japan). XRD analysis were carried out by means of a Bruker AXS (D8 Advance) (www.bruker.com) X-ray diffractometer. N_2 adsorption–desorption was established by Brunanuer–Emmett–Teller (BET) tests using Micromeritics Tristar 3000 gas adsorption analyzer. The distributions of pore size were estimatedvia BET theory and the Barrett–Joyner–Halenda (BJH) technique, the MCF were heated at 433 K in vacuum condition for about 5 h.

Biosensor fabrication

Mesoporous carbon foam (MCF) was prepared via the Pechini method [14] as described in the Electronic Supporting Material (ESM).

Then the GCE was polished with 1.0, 0.3 and 0.05 μm alumina slurry subsequently, then cleaned in bath sonication for 10 min and finally rinsed with distilled water. Afterwards, the MCF (2 mg) and Salep (2 mg) were mixed in water (1 mL) and the resulted mixture was sonicated for 30 min at room temperature to achieve a homogeneous and stable suspension containing 2.0 mg mL^{-1} MCF. Then, 4 mg Mb was dissolved in 1 mL phosphate buffer at pH 5.0 (the iso-electric point of Mb is at pH around 6.8 [20]), this biomolecule was positively charged inside the applied buffer (pH 5.0). Mb is adsorbed on the MCF surface essentially via electrostatic interactions. The resultant suspension was stirred at 4 °C for about 100 min which caused the Mb to adsorb on the MCF surface. For the fabrication of biosensor, 5 μL of suspension was casted on the cleaned GCE surface and dried in air. The biosensor (Mb/S-MCF/GCE) were washed with doubly distilled water and dried for 2 h at 4 °C. As a control, the similar protocols were applied to immobilize Mb on the bare GCE and electrode was modified with only Salep without presence of MCF.

Results and discussion

Choice of material

Salep is a flour made from the tubers of the orchid genus Orchis. It is widely consumed in beverages and desserts [19]. Carbon baesd nanocomposites, specially with biopolymers such as sodium alginate, chitosan and nafion, show high performance in electrochemical sensors and biosensors. Salep is similar to sodium alginate and chitosan in structure and properties, has advantageous properties such as cheapness, high availability, non-toxicity and gelling possessions. Salep is more preferred in comparison with chitosan and sodium alginateare due to its higher availability and lower price. Salep is available in many stores, confectioneries or even at some houses. MCF disperses in salep soloution homogeneusly. This composite, S-MCF, can provide an appropriate matrix for biomolecules immobilization.

Characterization of the mesoporous carbon foam

Morphology and size distribution of the MCF were characterized by means of TEM and FE-SEM methods. Figure 1a shows TEM image of the MCF in low. TEM image display the distinct pores with three dimensional channel organizations. TEM image with higher magnification is also shon in Fig. 1S (see ESM). Figure 1b, FE-SEM images of the MCF, shows the gritty with a three-dimensional and porous structure of the MCF. This porous structure of MCF, observed by TEM and SEM studies, can create an advanced substrate for entrapment of proteins and enzymes which is suitable material to be applied for biosensor area.

The XRD methods was applied for additional investigation of synthesized MC. Figure 2a indicated that the wide diffraction peak located at about 23°, a characteristic of carbon based materials, disclosed an amorphous nature for the MCF. This peak is in good agreement with previously published XRD reports for the carbon materials [28, 30].

Figure 2b shows the N_2 adsorption–desorption isotherms for MCF sample. Isotherm curves displayed characteristic IV shapes which is in accordance to the IUPAC arrangement. This curves confirms the mesoporous structure for prepared carbon structure [28]. According to isotherms and corresponding BET data, the estimated specific surface area and pore

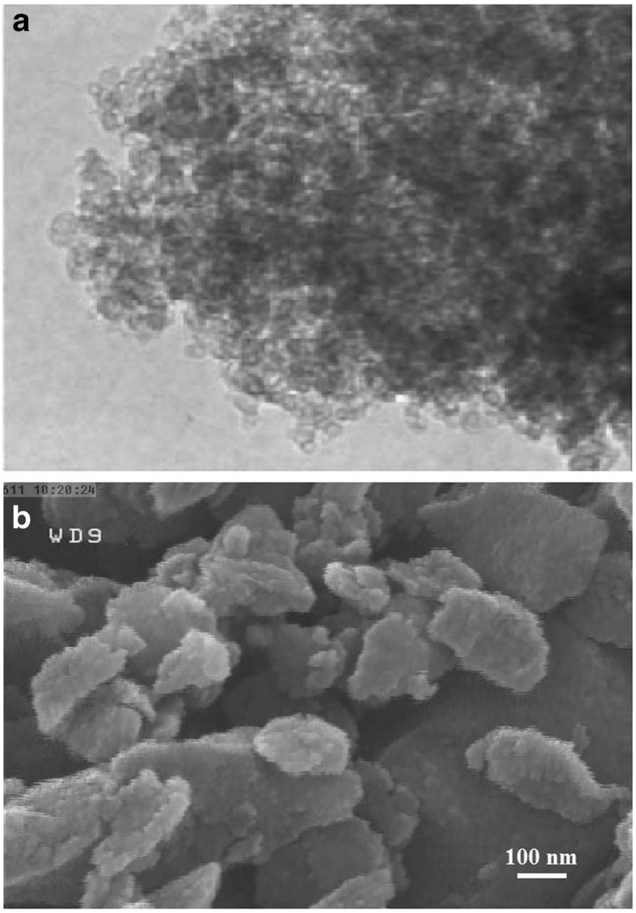

Fig. 1 **a** TEM image of the mesoporous carbon foam (MCF). **b** FE-SEM image of the MCF

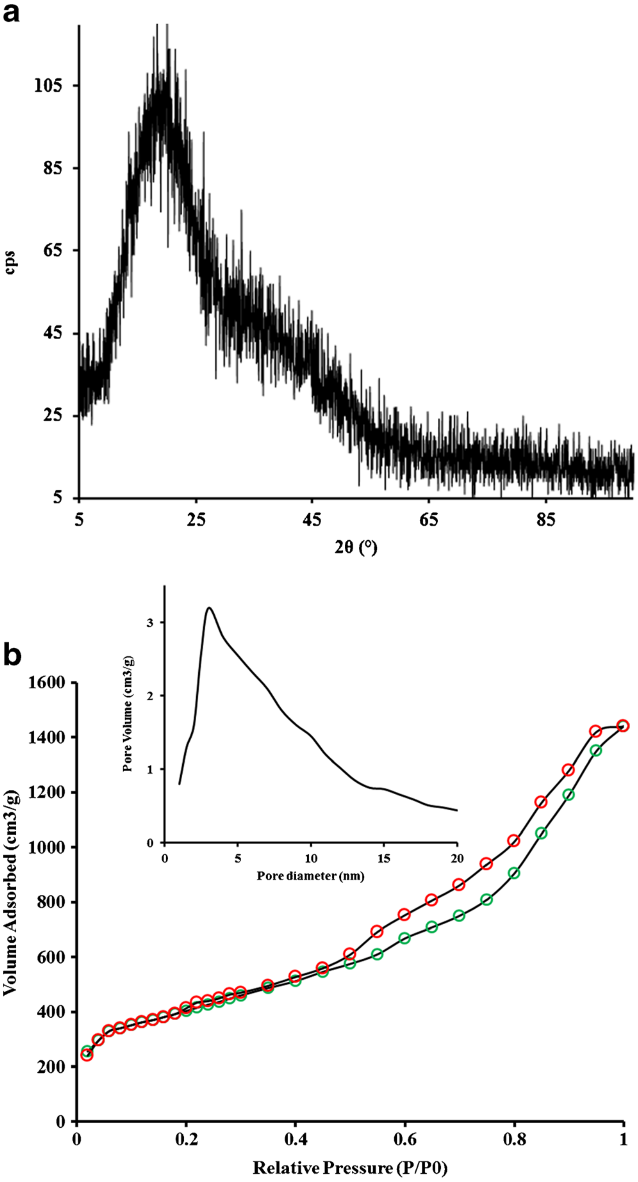

Fig. 2 **a** Nitrogen adsorption–desorption isotherms and (inset A) is the pore size distribution curve of prepared MCF sample. **b** XRD pattern of MCF

volume were obtained about 1435.65 m^2 g^{-1} and 1.2 cm^3 g^{-1} respectively. The average pore diameter of 6 nm was obtained from BJH method, which is in agreemnt with the TEM results.

The MC needs to be dispersed in aqueous or organic media to be used for biosensor application. MC drastically has tendency to aggregate in aqueous medium due to its poor interaction with water molecules. In contrast, MC can form homogeneous and stable suspension in Salep solution (see Fig. 2S). The binding between MC and nonpolar side-chains of hydrophilic biopolymers produced a stable dispersions of these nanoparticles in water which results in stable black suspension (stable more than 20 h) [16]. The mechanism is explained by breaking the MC aggregations as a result of forming complex with salep. It has proper biocompatibility which donates superior biocompatibility to the S-MCF composite. Furthermore, salep provided a suitable surface for immobilizing the Mb molecules and it is efficient dispersant for carbon materials.

Direct electrochemistry of Mb/S-MCF/GCE

Figure 3 illustrates the cyclic voltammograms of Mb/S-MCF/GCE, Mb/S/GCE and Mb/GCE in N_2 saturated 0.1 M phosphate buffer at pH 7.0. There are no clear redox signals for Mb/GCE (Fig. 3a) and Mb/Salep modified electrode (Fig. 3b). Figure 3a shows the cyclic voltammogram of Mb/S-MCF/GCE at the sweep rate of 100 mVs^{-1} that represents a distinct and quasi-reversible redox peak at −0.32 V (E_{pc}) and −0.23 V (E_{pa}), and the peak-to-peak separation is found to be around 90 mV. These results corresponds to the characteristics of Mb Fe(III)/Fe(II) redox center [21], which show a fast DET for Mb enmeshed into the composite film. The improved DET of Mb incorporated in S-MCF composite film compared to Mb/S/GCE and Mb/GCE can arise from the critical role of MCF as a promoter component to facilities DET between Mb and electrode surface. This results confirms that the (i) MCF has an absolute influence on the kinetics of biosensor, (ii) MCF provides a suitable matrix for electron transfer of Mb and (iii) MCF creates a promising mesoporous environment to prevent denaturation of Mb [20, 21]. In addition, the anodic and cathodic peaks are closely symmetric and the oxidation and reduction signal heights are approximately equal (see, Fig. 3c). These results indicate the fully transformation of Fe(III) species in center of Mb to Fe(II) species in the reverse scan rate confirming an ideal behavior for immobilized hemoprotein.

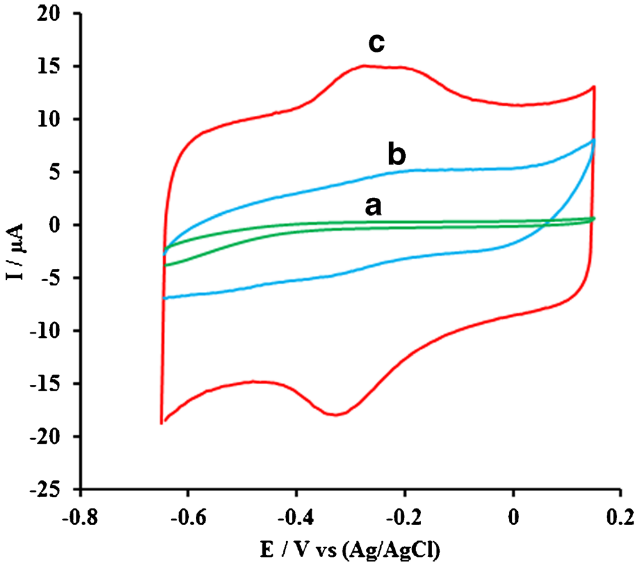

Fig. 3 CVs of Mb/GCE (**a**), Mb/S/GCE (**b**), and Mb/S-MCF/GCE (**c**) in 0.1 M phosphate buffer (pH 7.0) at scan rate of 100 mV s^{-1}

The influence of scan rate on the signals was examined to explore the features of Mb at the Mb/S-MCF/GCE by using various sweep rates (please see ESM). More investigates have been performed to study the effect of pH on the electrochemical behavior of Mb/S–MCF/GCE. The cyclic voltamograms of Mb/S-MCF/GCE recorded in buffers for pH ranging from 3.0 to 9.0. Reslts indicated that both reduction and oxidation potential peaks shift negatively when the pH increases (See Fig. 3S in ESM). This consequences proves that the process is along with the proton transfer. Moreover, the slopes of E_{pa} and E_{pc} vs. pH are −51.8 mV. pH $^{-1}$ and −50.3 mV. pH $^{-1}$ respectively. The obtained slopes for E_{pa} and E_{pc} are very close to the Nernstian slope of −59 mV for electrochemical procedures involving the equal number of electrons and protons [22]. The slope of −59 mV represented the process of single-electron transfer combined with one proton as follows [23]: Mb [Fe (III) + e^- + H^+ ↔ Mb [H^+-Fe (II)].

From Fig. 3S it can be understood that the biosensor shows better behavior in pHs of 3 and 7 compared to other pHs. So, pH 7.0 (pH of biological medium) was used in all experiments.

Electro-catalytic reduction of H_2O_2 at Mb/S-MCF/GCE

The DET of Mb was improved at the S-MCF nanocomposite substrate as entrapped Mb into the S-MCF composite possess the enzymatic performance. The Mb/S-MCF provides a suitable platform for fabricating the third-generation of biosensor for H_2O_2 detection since the heme is perfectly recognized as an effective electro-catalyst toward H_2O_2 reduction. The behavior of biosensor to electro-reduction of H_2O_2 was studied here to further explore the activity of entrapped Mb into S-MCF composite film.

Figure 4 displays the cyclic voltammogram of Mb/S-MCF/GCE in the presence of H_2O_2 (Fig. 4a) and the absence of H_2O_2 (Fig. 4b and c). The evaluation of these curves reveals that after adding H_2O_2, the reduction current increases accompanied by decreasing oxidation peak. These performance confirms the electro-catalytic activity of the immobilized Mb on S-MCF toward the

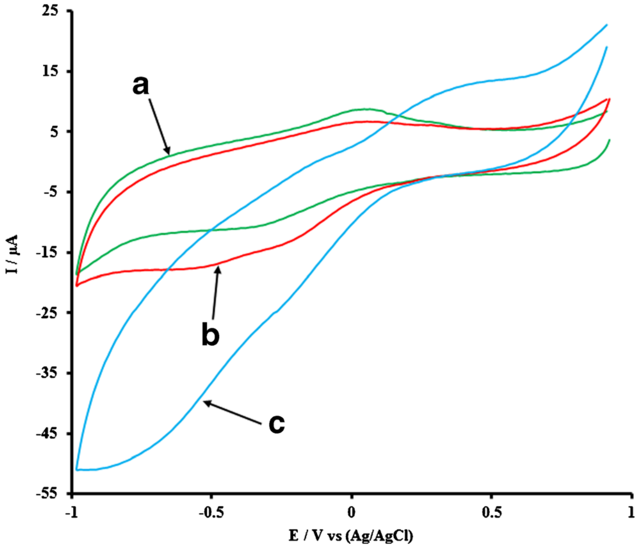

Fig. 4 Cyclic voltammetry responses of Mb/S-MCF/GCE in the presence of 0 (**a**), 0.5 (**b**) and 1 (**c**) mM of H_2O_2 in phosphate buffer (pH 7.0) at scan rate of 100 mV s^{-1}

reduction of H_2O_2 [5, 20]. Additionally, this result might be explained by bio-activity of the immobilized Mb on the S-MCF medium which exhibits suitable catalytic performance to the H_2O_2 electro-reduction. The electro-reduction mechanism can be explained as follows [24]:

$$\mathrm{Mb}\,heme\,\mathrm{Fe(III)} + \mathrm{H}^+ + \mathrm{e}^- \rightarrow \mathrm{Mb}\,heme\,\mathrm{Fe(II)}\,2\,\mathrm{Mb}\ heme\ \mathrm{Fe(II)}$$
$$+ \mathrm{H_2O_2} + 2\mathrm{H}^+ \rightarrow 2\,\mathrm{Mb}\ heme\ \mathrm{Fe(III)} + 2\,\mathrm{H_2O}\,\mathrm{Mb}\ heme\ \mathrm{Fe(III)}$$
$$+ \mathrm{H}^+ + \mathrm{e}^- \rightarrow \mathrm{Mb}\ heme\,\mathrm{Fe(II)}$$

Analytical performance of the Mb/S-MCF/GCE toward determination of H_2O_2

The Mb/S-MCF/GCE was applied to detect H_2O_2 concentration. For this purpose, the amperometric experiments were accomplished in an electrochemical cell containing 0.1 M phosphate buffer, stirred at 1000 rpm in the applied potential of −0.2 V. Figure 5 displays the amperometric signals of Mb/S-MCF/GCE to the additions of H_2O_2 concentrations. The reaction at the surface of biosensor reaches to a dynamic steadiness rapidly within the time period of 2–5 s (immediately after each addition of the H_2O_2). As it is presented in inset of Fig. 5, the linear range is from 1.0 to 80.0 µM and the linear equation was obtained as follow: I (µM) = −0.3774 $C_{\text{Hydrogen peroxide}}$ (µM) − 1.8454 ($R^2 = 0.9994$). The limit of detection for the methode is about 180 nM ($S/N = 3$). Also, the RSD was realized to be about 2.9%, which demonstrate the repeatability/reproducibility of the method. The sensitivity was estimated to be 2.2 µA. µM. cm-2. Compared to the other electrochemical H_2O_2 sensors and biosensors, the Mb/S-MCF/GCE shows acceptable performance (Table 1).

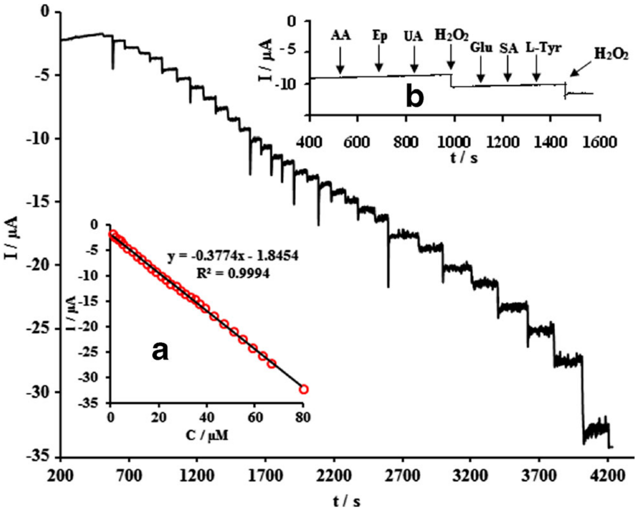

Fig. 5 Amperometric responses of Mb/S-MCF/GCE to stepwise addition of H_2O_2 (1.0 to 80 µM) in 0.1 M phosphate buffer solution (pH 7.0) in the conditions of −0.2 V constant potential and rotation speed of 1000 rpm. Inset a is the plot of current against H_2O_2 concentration. (Inset b) Currenttime responsesof the Mb/S-MCF/GCE to 10 µM H_2O_2 (first and last additions) and 0.1 mMAA, Dp, UA, Glu, CA and L-Tyr under the conditions of −0.2 V applied potential and rotation speed of 1000 rpm

The Michaelis-Menten constant (K_M) can be estimated from the Line weaver-Burk equation ($I_{ss} = I_{max}$ - K_M (I_{ss}/C_{ss}), where I_{ss} is the steady-state current, C_{ss} is the concentration of analyte, and I_{max} is the determined signal measured in saturated H_2O_2 concentration. The K_M for Mb/S-MCF/GCE was calculated to be about 0.22 mM confirmaing the high affinity of the biosensor to H_2O_2 [35].

Biosensor reproducibility, repeatability, and stability

The investigation of reproducibility, repeatability and stability of a biosensor are actually essential for its application in real sample analysis. Therefore, these parameters in the optimal conditions need to be examined for the biosensor. Reproducibility of the Mb/S-MCF/GCE was estimated in 0.1 M phosphate buffer at pH 7 containing 10 µM H_2O_2 for four detections. The relative standard deviation (RSD) was found to be about 2.6%. Moreover, to obtain repeatability of the biosensor, three independent Mb/S-MCF/GCE were applied to estimate the concentration of 10 µM H_2O_2. The RSD was found to be about 3.1%, which reveals the outstanding repeatability for biosensor production method. To observe the long-term stability of Mb/S-MCF/GCE, firstly the response of biosensor was obtained in 10 µM H_2O_2 solution and then the electrode was washed with doubly distilled water and reserved in 0.1 M buffer at pH 7. After 1 and 3 weeks, the decreased signal were found to be about 2.1% and 5.9% respectively. These results

Table 1 Comparison of several modified electrodes for H_2O_2 sensing

Electrode	Modifier	Linear range (μM)	Limit of detection (μM)	Reference
Glassy carbon	PEDOT/PB nanocomposite	0.5–839	0.16	[25]
Glassy carbon	Hb/PANI/SnO2NF/CH	2–160	0.5	[26]
Glassy carbon	graphite-CS/PtNP composite	0.25–2890	0.066	[27]
Glassy carbon	CNH/PAP composite	50–8000	3.6	[28]
Glassy carbon	Nafion/NPC-CB composite	not given	1.2	[29]
Glassy carbon	RGO/Pt-Ag NPs	5–150	0.04	[30]
Glassy carbon	AgPdNPs	20–500	0.52	[31]
Glassy carbon	G/rGO/polyHm	9.9–50	8.86	[32]
Glassy carbon	Pd-CoCNTs	1–110	0.3	[33]
Glassy carbon	Hb-AgNPs-PAMAM	6–91	4.9	[34]
Glassy carbon	Mb/S-MCF	1–80	0.18	this work

proves that this biosensor has respectable stability, repeatability, and reproducibility.

Interference studies and real sample analysis

Some compounds possibly presented in biological samples were explored for their interference on the amperometric detection of 10 μM H_2O_2. Ascorbic acid (AA), dopamine (Dp), uric acid (UA), Glucose (Glu), salicylic acid (SA), and L-tyrosine (L-Tyr) interfering effects on the amperometric signal of H_2O_2 was observed and represented in insert b of Fig. 5. The biosensor displays no amperometric current toward 0.1 mM of each cited species. Nevertheless, a distinct signal was appeared after addition of 10 μM H_2O_2. The selectivity of this biosensor were aroused from the low applied potential (−0.2 V) in amperometric experiments.

To end, disinfected fetal bovine serum (FBS) was used as real sample in order to evaluate the capability of the biosensor in biological sample analysis. FBS was pretreated using an arrangement of membrane filtration and centrifugation. The filtration process performs through the application of three sterilizing grade membrane filters (0.1 μm), serially. Next, the solution of methanol (5% (*v*/v)) was added to the treated samples and centrifuged under 7500 rpm for around20 min. Each sample was diluted with 0.1 M buffer (pH 7) and spiked with the standard solutions of H_2O_2 ranging from 1.0 to 10.0 μM. Amperometric method was used for evaluation of H_2O_2 content in samples. The consequences have been reported in Table 2.The average recovery was calculated to be around 97.3%. Above results suggest that the S-MCF composite performs as an ideal substrate for immobilizing Mb and the biosensor is reliable and effective in determination of H_2O_2 in biological samples.

Conclusion

The salep solution shows high ability to disperse MCF produced via Pechini technique. This carbon nanocomposite is an appropriate matrix for Mb immobilization. MCF dispersing in salep increased its biocompatibility and considerably assisted the bio-electrochemical capability of MCF. The DET of Mb entrapped into S-MCF composite film was principally improved.The biosensor productively sensed H_2O_2 with short response time (2–5 s), extensive linear range and low detection limit. This biosensor reveals good sensitivity, standing stability, also acceptable repeatability and reproducibility. In overal, the bio-device organized in this assay is promising for the future development of sensors and biosensors. Perhaps the storage conditions of the biosensor is one of its limitations, which is done by placing it in a buffer and holding in the refrigerator.

Table 2 Results of recovery analysis of H_2O_2 in FBS samples ($n = 5$)

No.	Added (μM)	Found (μM)	Measured current (μA)	Recovery (%)	Average recovery (%)	RSD (%, n = 5)
1	1.0	1.02	0.24	102	97.33	3.3
2	2.0	1.88	0.45	94		2.85
3	3.0	2.88	0.67	96		2.0

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Compliance with ethical standards The author(s) declare that they have no competing interests.

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