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Fabrication of gallium hexacyanoferrate modified carbon ionic liquid paste electrode for sensitive determination of hydrogen peroxide and glucose

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ABSTRACT

Gallium hexacyanoferrate (GaHCFe) and graphite powder were homogeneously dispersed into n-dodecylpyridinium hexafluorophosphate and paraffin to fabricate GaHCFe modified carbon ionic liquid paste electrode (CILPE). Mixture experimental design was employed to optimize the fabrication of GaHCFe modified CILPE (GaHCFe-CILPE). A pair of well-defined redox peaks due to the redox reaction of GaHCFe through one-electron process was observed for the fabricated electrode. The fabricated GaHCFe-CILPE exhibited good electrocatalytic activity towards reduction and oxidation of H_2O_2. The observed sensitivities for the electrocatalytic oxidation and reduction of H_2O_2 at the operating potentials of +0.8 and −0.2 V were about 13.8 and 18.3 mA M^{-1}, respectively. The detection limit (S/N = 3) for H_2O_2 was about 1 μM. Additionally, glucose oxidase (GOx) was immobilized on GaHCFe-CILPE using two methodology, entrapment into Nafion matrix and cross-linking with glutaraldehyde and bovine serum albumin, in order to fabricate glucose biosensor. Linear dynamic rage, sensitivity and detection limit for glucose obtained by the biosensor fabricated using cross-linking methodology were 0.1–6 mM, 0.87 mA M^{-1} and 30 μM, respectively and better than those obtained (0.2–6 mM, 0.12 mA M^{-1} and 50 μM) for the biosensor fabricated using entrapment methodology.

1. Introduction

Simple and economical fabrication of an easy-to-use sensor with high electroanalytical performance towards analyte detection is an attractive area in electroanalysis. Carbon past electrode (CPE) is one of the oldest carbon composite electrode that more than a half century passes since its invention in 1958. For the fabrication of a traditional CPE, graphite powder is hand mixed with a pasting liquid such as paraffin or silicon oils. But, a series of innovations [1] has been applied for the fabrication of new type CPE with the enhanced electrochemical performance. The use of carbon nanotubes (CNTs) instead of graphite powder [2] or the use of room temperature ionic liquids (RTILs) [3] instead of paraffin or silicon oils are two examples of such innovations. Attractive features such as ease of fabrication and regeneration, low background current, stable response and very low ohmic resistance have been cited for CPEs. These properties can be combined with the distinct properties of ILs, e.g. high chemical and thermal stability, relatively high ionic conductivity, negligible vapor pressure and wide electrochemical window, when ILs are used as the binder for the fabrication of new type CPE. It has been shown that the electrochemical performance of this new type carbon paste electrode, so called carbon ionic liquid electrode (CILE), has significantly improved compared to CPE [4–6]. Also, it has been shown that the electrochemical properties of the biomolecules such as nicotinamide adenine dinucleotide (NADH) enhance on CILE. So, it seems that CILE is a good candidate for the construction of new type biosensors [5,7].

Design and fabrication of a sensitive hydrogen peroxide sensor are attractive perspectives for many researchers. Also, it is a fundamental part for the construction of a biosensor in which the production or consumption of hydrogen peroxide is measured. Among the reported hydrogen peroxide sensors, the most interesting are Prussian blue (PB)-based sensors in which PB acts as an electron transfer mediator. Using a PB-based sensor, detection of hydrogen peroxide is performed through its electrocatalytic reduction at the reduced overpotential e.g. 0.0 vs. Ag|AgCl|KCl_{sat}. So, at this reduced overpotential the detection is selective for hydrogen peroxide even in the presence of oxygen and many other electroactive compounds which are usually present in real samples [8]. Prussian blue (ferric hexacyanoferrate, FeHCFe) and its analogs are the prototype of a number of polynuclear transition metal hexacyanometalates. Many efforts have been devoted to fabricate metal hexacyanoferrate (MHCFe) modified electrodes and also to show their importance and possible applications in electroanalysis [9]. To the best of our knowledge, there is only one report detailing the electrochemical behavior and performance of gallium hexacyanoferrate as electrocatalyst for H_2O_2 [10]. In the mentioned study, gallium

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hexacyanoferrate modified CPE has been used for the detection of H_2O_2 on the basis of its electrocatalytic activity towards hydrogen peroxide reduction.

In this study, GaHCFe was incorporated into a carbon ionic liquid paste electrode (CILPE) composed of n-dodecylpyridinium hexafluorophosphate ($[C_{12}Py][PF_6]$) and paraffin as the pasting agents. Mixture experimental design [11] was used to find the optimal weight ratio of graphite powder, $[C_{12}Py][PF_6]$ and paraffin for the sensor fabrication. The electrochemical properties of GaHCFe-CILPE and its electrocatalytic activity towards hydrogen peroxide were studied and the analytical features of the sensor for the amperometric determination of hydrogen peroxide were evaluated. Thereafter, glucose oxidase (GOx) was immobilized on the surface of GaHCFe-CILPE to fabricate a glucose biosensor. Two immobilization methodologies, cross-linking with glutaraldehyde (GLU) and bovine serum albumin (BSA) and entrapment into Nafion were applied and the analytical features of the fabricated biosensors for the amperometric determination of glucose were evaluated.

2. Experimental

2.1. Chemicals

All chemicals were of analytical reagent grade and used without further purification. High purity graphite powder (particle size <0.1 mm), n-dodecyl iodide, pyridine, ammonium hexafluorophosphate, ascorbic acid, $Ga(NO_3)_3 \cdot 9H_2O$, CH_3COONa, H_3PO_4, KOH, KCl, HCl, $K_3[Fe(CN)_6]$, $K_4[Fe(CN)_6]$ and H_2O_2 (30%) were purchased from Merck (Darmstadt, Germany). Nafion perfluorinated ion-exchange (5% solution in 90% light alcohol), ethanol (97%), glutaraldehyde (GLU, grade I, 25%), bovine serum albumin (BSA, fraction V, minimum 96%) were obtained from Fluka (Buchs, Switzerland). Glucose oxidase (GOx, EC 1.1.3.4, type VII from *Aspergillus niger*, 221 U mg^{-1}) and D(+)-glucose (97%) were obtained from Sigma (St. Louis, MO, USA). Double distilled water was used throughout the experiments.

$[C_{12}Py][PF_6]$ was prepared according to the method reported previously for the preparation of n-octylpyridinium hexafluorophosphate [12]. GaHCFe was synthesized using the method reported previously [10]. In brief, 20 mL solution containing 0.1 M $K_4[Fe(CN)_6]$ and 1 M KCl was added dropwise to a 20 mL solution of 0.1 M $Ga(NO_3)_3 \cdot 9H_2O$ (pH = 2) during 3 h under rigorous stirring. The precipitate was then collected by filtration and washed thoroughly with 1 M KCl (20 mL × 3) and double distilled water (20 mL × 3). Afterward, the precipitate was dried by P_2O_5 in a vacuum desiccator at ambient temperature for two days.

Nine milligrams of GOx was dissolved in 400 μL of acetate buffer (0.05 M, pH = 5.5) to prepare a 5 U μL^{-1} of GOx working solution. Working solutions of BSA and GLU at the required concentrations were prepared in water. 0.05 M KH_2PO_4 and 0.1 M KCl were used to prepare phosphate buffer solution (PBS) and its pH was adjusted to 6.4 using KOH solution. Stock solutions of glucose (0.5 M) and H_2O_2 (0.2 M) were prepared in water and stored at 4 °C when not in use. The stock solution of glucose was allowed to mutarotate at room temperature overnight before use. Standard working solutions of glucose and H_2O_2 were prepared freshly by diluting their stock solutions with PBS (0.05 M KH_2PO_4 and 0.1 M KCl (pH = 6.4)).

2.2. Apparatus

Electrochemical measurements were performed using an Autolab potentiostat-galvanostat model PGSTAT30 (Metrohm-Autolab B.V., Utrecht, The Netherlands) with a conventional three-electrode setup. A GaHCFe-CILPE, an $Ag|AgCl|KCl_{sat}$ electrode and a platinum wire were used as the working, reference and auxiliary electrodes, respectively. The working potential was applied to the working electrode in the standard way using the potentiostat and the output signal was acquired using Autolab NOVA software. A Metrohm 691 pH meter (Metrohm A.G., Herisau, Switzerland) was used for pH adjustment. Fourier transform infrared (FTIR) spectroscopy was performed on a Bruker vector-22 spectrophotometer (Bruker Optik GmbH., Germany). Design expert program (DX8, trail version) was downloaded from the Stat-Ease software web site (Stat-Ease, Minneapolis, MN, USA) [13]. DX8 was employed to find the optimal weight ratio of materials used for the sensor fabrication.

2.3. Fabrication of GaHCFe-CILPE

Appropriate amounts of graphite powder, $[C_{12}Py][PF_6]$, paraffin and GaHCFe were dispersed in acetone. The resulting mixture was then hand mixed using a mortar and pestle for about 30 min under ambient conditions. A portion of the resulting paste was packed firmly into a glass tube (3.2 mm i.d., A = 0.0804 cm^{-2}). The surface of the prepared GaHCFe-CILPE was wiped and smoothed with weighing paper. The electric contact was established using a copper wire through the back. For the preliminary electrochemical studies, three GaHCFe modified CPE, CILE and CILPE were prepared through a similar procedure. GaHCFe-CPE was prepared using graphite powder, paraffin and GaHCFe with a weight ratio of 65:25:10 (w%). GaHCFe-CILE was prepared using graphite powder, $[C_{12}Py][PF_6]$ and GaHCFe with a weight ratio of 65:25:10 (w%). GaHCFe-CILPE was prepared using graphite powder, $[C_{12}Py][PF_6]$, paraffin and GaHCFe with a weight ratio of 60:20:10:10 (w%). The prepared GaHCFe modified electrodes were activated in PBS by cycling the potential from −0.2 to +0.8 V at a scan rate of 100 mV s^{-1} for 20 times.

2.4. Fabrication of glucose biosensors

Two classic methods of immobilization, A) cross-linking of GOx with GLU and BSA and B) entrapment of GOx into Nafion layer, were used to immobilize GOx on top of the GaHCFe-CILPE.

A) A final volume of 5 μL aqueous solution containing GOx (1.2 U), GLU (0.5% (v/v)) and BSA (0.8% (w/v)) was placed on top of the GaHCFe-CILPE. The fabricated biosensor (GaHCFe-CILPE\GOx-BSA-GLU) was then allowed to dry at room temperature for 30 min.

B) 200 μL Nafion solution (5%) was mixed with 795 μL ethanol (99.7%) and 5 μL NH_4OH aqueous solution (25%) to prepare neutralized Nafion solution (1%). A final volume of 5 μL aqueous solution containing GOx (0.4 U) and Nafion (0.7% (v/v)) was placed on top of the GaHCFe-CILPE. The fabricated biosensor (GaHCFe-CILPE\GOx-Nafion) was then left to dry at room temperature for 20 min.

3. Results and discussion

Cyclic voltammetry was performed using the prepared GaHCFe modified CPE, CILE and CILPE as the working electrode in PBS (pH = 6.4) at the scan rate of 100 mV s^{-1}. As shown in Fig. 1, the formal potential ($E^{\circ\prime} = 1/2(E_{pa} + E_{pc})$, the mean value of the anodic and cathodic peak potentials) for GaHCFe-CILPE was about +0.216 V vs. $Ag|AgCl|KCl_{sat}$ and less than those observed for GaHCFe-CPE (+0.247 V) and GaHCFe-CILE (+0.252 V). Also, the value of peak potential separation ($\Delta E_p = E_{pa} - E_{pc}$) for GaHCFe-CILPE was about +0.072 V and much lower than those observed for GaHCFe-CPE (+0.293 V) and GaHCFe-CILE (+0.288 V). The ratio of the anodic peak current (i_{pa}) to its corresponding cathodic peak current (i_{pc}) for GaHCFe-CILPE and GaHCFe-CPE was about 0.8 and significantly different from that observed for GaHCFe-CILE (0.3). The ratio of i_{pc} to its charging current intensity (i_c) i.e. i_{pc}/i_c for GaHCFe-CILPE was about 10 and higher than that observed for GaHCFe-CPE (about 5). Therefore, GaHCFe-CILPE which exhibited improved electrochemical behavior in terms of $E^{\circ\prime}$, ΔE_p and i_{pc}/i_c was selected for the fabrication of new type H_2O_2 sensor and glucose biosensor. It seems that not only the mixture of IL and paraffin presents the advantages of both IL and paraffin but

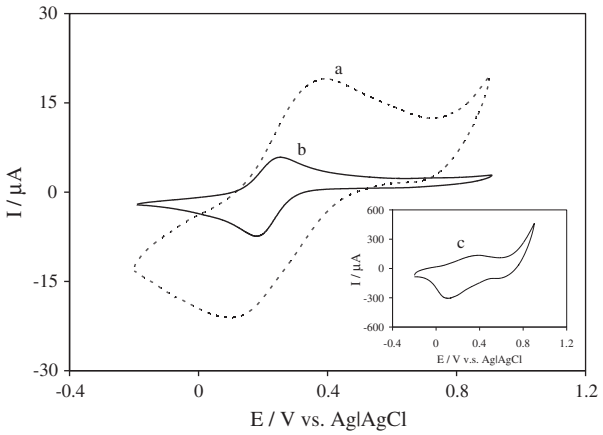

Fig. 1. Typical cyclic voltammograms obtained for GaHCFe-CPE (a), GaHCFe-CILPE (b) and GaHCFe-CILE (c) in PBS (pH = 6.4) at the scan rate of 100 mV s^{-1}.

also the synergistic effect of them in their mixture improves the electrochemical behavior of GaHCFe-CILPE.

3.1. Optimizing the fabrication of GaHCFe-CILPE

Mixture experimental design [11,14] in which each component of a mixture is considered as a factor was exploited for the optimization of GaHCFe-CILPE fabrication. In this design the ratio of factors (components present in the mixture i.e. controlled variables) must add up to 1 or 100%. It means, the amount of each factor does not matter but its ratio in total is important.

The weight ratio of GaHCFe for GaHCFe-CILPE fabrication was kept constant at 0.1 (10%) during the course of optimization. Then, on the basis of three-factor mixture design the ratios of graphite powder, $[C_{12}Py][PF_6]$ and paraffin (factors) were varied with considering their experimental constrains to prepare nine GaHCFe-CILPEs. Additionally, three electrodes were selected randomly among of the prepared 9 electrodes for replication. The prepared 3 replicated electrodes were examined with the other 9 electrodes to evaluate the experimental random error and to compare the experimental random error with the lack of fit of the mathematical models. The compositions of the prepared 12 GaHCFe-CILPEs are listed in Table 1. As seen in Table 1, the applied minimum and maximum weight ratios (constraints) for graphite powder are 0.5 (50%) and 0.7 (70%), respectively. The use of graphite powder less than 0.5 produced a loose composite and more than 0.7 produced a dry composite which they did not packed firmly into the glass tube. The applied minimum weight ratios for $[C_{12}Py][PF_6]$ and paraffin were selected 0.05 (5%) on the basis of the results obtained in the preliminary studies.

Thereafter, cyclic voltammetry was carried out using the prepared 12 GaHCFe-CILPEs as the working electrode in PBS (pH = 6.4) at the scan rate of 100 mV s^{-1}. The results showed that the ratios of i_{pa} to its corresponding i_{pc} for the studied electrodes were almost constant (between 0.83 and 0.78). But, the peak current intensity and the peak potential separation value were dependent on the electrode composition (factors). So, the values of $-\log(|i_{pc}|)$ and ΔE_p listed in Table 1 were selected as two analytical response criteria to find the optimal weight ratios of the components (factors) for GaHCFe-CILPE fabrication.

Two regression equation models were found between the factors and two analytical response criteria i.e. $-\log(|i_{pc}|)$ and ΔE_p. The obtained regression equations are presented in Eqs. (1) and (2):

$$\Delta E_p = 0.60A + 1.28B + 1.97C - 3.26AB - 4.59AB - 2.47BC \quad (1)$$

$$-\log\left(\left|i_{pc}\right|\right) = 2.98A + 6.65B + 9.22C \quad (2)$$

in which A, B, and C (three factors) are the weight ratio of graphite powder, $[C_{12}Py][PF_6]$ and paraffin in the mixture, respectively. The calculated values for $-\log(|i_{pc}|)$ and ΔE_p using the proposed regression equation models and their response surface plots are presented in Table 1 and Fig. 2, respectively. The analysis of variance (ANOVA) was applied to the experimental and calculated data (Table 1). The results showed that the effects of factors (weight ratio of components in the mixture) on two analytical responses were significant and the differences between the experimental and calculated data were in the range of experimental random error (P-Model < 0.05 and P-LOF > 0.05).

Response trace plot [14] was employed to show the effect of each factor on two response criteria (Fig. 3). In this methodology by the use of the proposed regression equation models, change in the calculated response due to the change of the proportion of a single component as moving away from a reference mixture (centroid of the experimental region i.e. A = 0.60 (60%), B = 0.15 (15%) and C = 0.15 (15%)) is measured while the relative proportions of the other components are kept constant [15]. As shown in Fig. 3, ΔE_p increases with increasing the proportion of graphite powder but passes over a minimum with increasing the proportions of $[C_{12}Py][PF_6]$ and paraffin. Also, the value of $-\log(|i_{pc}|)$ decreases with increasing the proportion of graphite powder and increases with increasing the proportion of paraffin and slightly with increasing the proportion of $[C_{12}Py][PF_6]$.

As shown in Figs. 2 and 3 the value of $-\log(|i_{pc}|)$ is a linear function, but ΔE_p value is a non-linear function of three factors. So, multiple response surface methodology [13] and desirability function [16,17]

Table 1
The composition of the prepared 12 GaHCFe-CILPEs using thee-factor mixture design at fixed ratio of GaHCFe (0.1) and their experimental and calculated analytical responses.

Electrode (no.)	Graphite (A)	$[C_{12}Py][PF_6]$ (B)	paraffin (C)	$-\log(\|i_{pc}\|)^a$	$\Delta E_p{}^a$ (V)	$-\log(\|i_{pc}\|)^b$	$\Delta E_p{}^b$ (V)
1	0.7	0.05	0.15	3.7144	0.1660	3.8007	0.1669
2	0.7	0.05	0.15	3.7721	0.1530	3.8007	0.1669
3	0.7	0.10	0.10	3.5560	0.1960	3.6722	0.1726
4	0.7	0.15	0.05	3.4510	0.1820	3.5436	0.1907
5	0.6	0.05	0.25	4.8794	0.1090	4.4252	0.1010
6	0.6	0.15	0.15	4.5346	0.0770	4.1681	0.0867
7	0.6	0.25	0.05	3.7375	0.1240	3.9111	0.1222
8	0.5	0.05	0.35	5.0325	0.1220	5.0496	0.1269
9	0.5	0.05	0.35	4.6976	0.1300	5.0496	0.1269
10	0.5	0.20	0.20	4.6737	0.0690	4.6641	0.0673
11	0.5	0.35	0.05	4.3251	0.1130	4.2785	0.1190
12	0.5	0.35	0.05	4.2708	0.1250	4.2785	0.1190

[a] Experimental data.
[b] Calculated data using the proposed regression equation models.

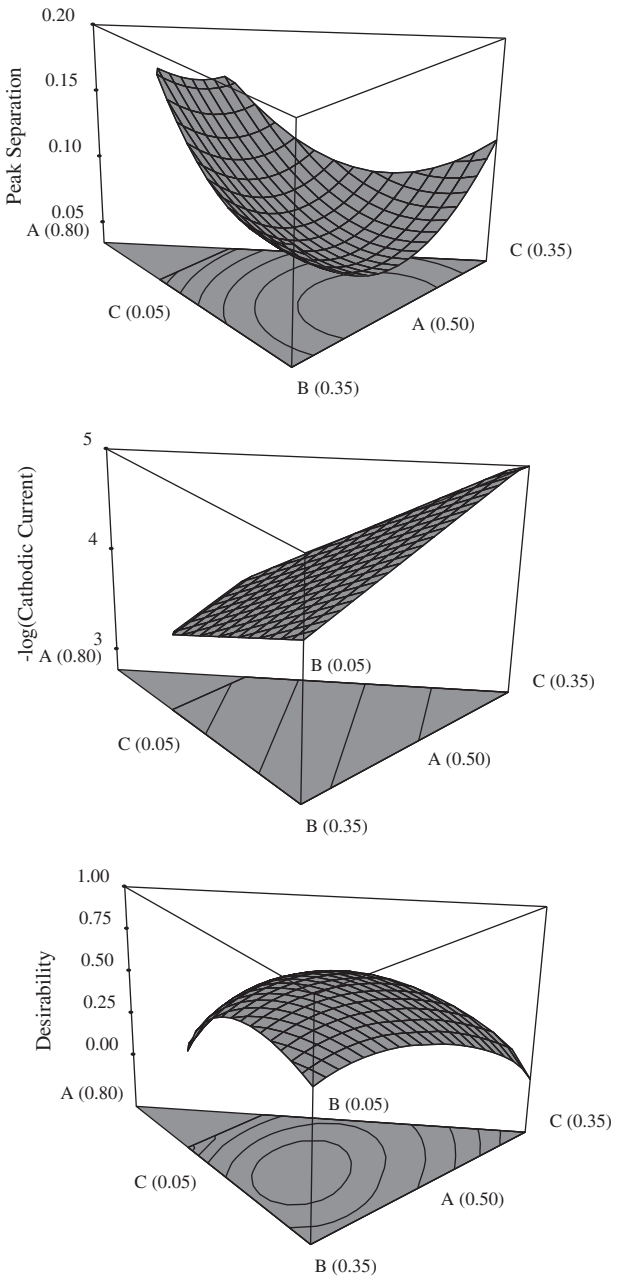

Fig. 2. The response surface plots for ΔE_p (V), $-\log(|i_{pc}|)$ and desirability in the space of three factors, weight ratios of graphite powder (A), $[C_{12}Py][PF_6]$ (B) and paraffin (C). Conditions: cyclic voltammetry in PBS (pH = 6.4) at the scan rate of 100 mV s^{-1}.

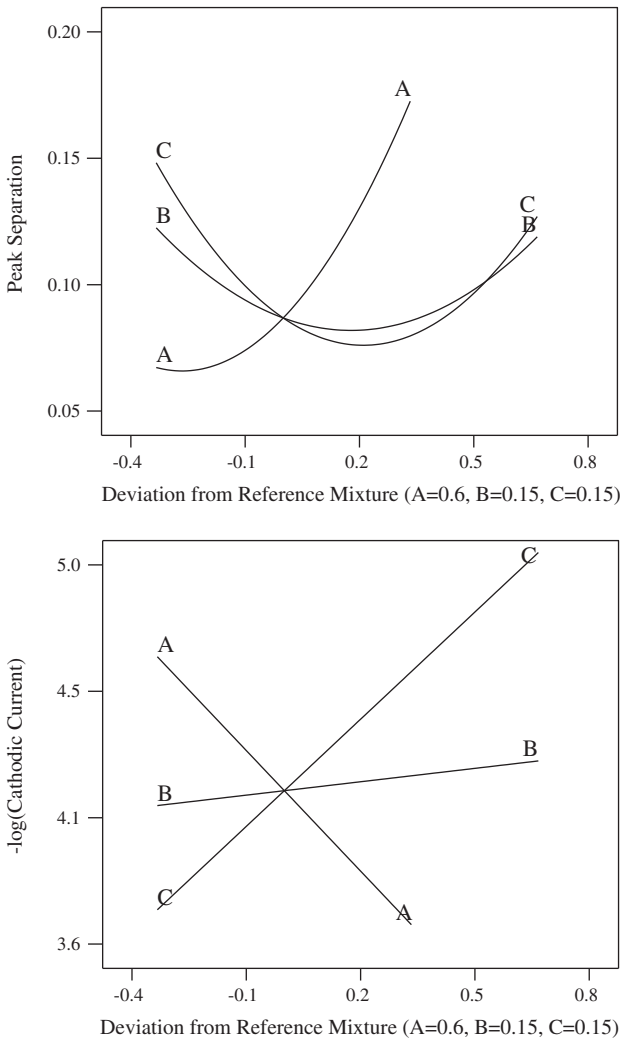

Fig. 3. Response trace plots for the calculated ΔE_p and $-\log(|i_{pc}|)$ using the proposed regression equation models due to the change of the proportion of a single component as moving away from a reference mixture (graphite powder (A) = 0.6, $[C_{12}Py][PF_6]$ (B) = 0.15 and paraffin (C) = 0.15).

were applied on two analytical responses, simultaneously to find the optimal weight ratios of the components which provided minimum $-\log(|i_{pc}|)$ (maximum cathodic peak current intensity) and ΔE_p values. As shown in Fig. 2, the maximum desirability for GaHCFe-CILPE fabrication is achieved at the optimal weight ratio of 0.59 (59%) for graphite powder, 0.19 (19%) for $[C_{12}Py][PF_6]$ and 0.12 (12%) for paraffin. Therefore, GaHCFe-CILPE was fabricated using graphite powder, $[C_{12}Py][PF_6]$, paraffin and GaHCFe with a weight ratio of 59:19:12:10 (w%), respectively for further studies.

3.2. Electrochemical characteristics of GaHCFe-CILPE

Electrochemical behavior of the optimized GaHCFe-CILPE was explored by cyclic voltammetry in PBS (pH = 6.4) at different scan rates and in potential range between −0.2 and +0.6 V. A pair of well-defined redox peaks was observed in the recorded cyclic voltammograms. The observed redox peaks was attributed to the redox reaction of GaHCFe in the presence of K^+ according Eq. (1):

$$Ga\left[Fe^{(III)}(CN)_6\right] + e^- + K^+ \leftrightarrows KGa\left[\left(Fe^{(II)}(CN)_6\right]\right. . \quad (3)$$

The observed values for $E^{\circ\prime}$ and ΔE_p were about +0.216 and +0.073 V at the scan rate of 100 mV s^{-1}. The value of ΔE_p increased linearly with increasing the scan rate in the range between 25 and 700 mV s^{-1}. Based on the Laviron theory [18] and assuming the negligible effect of orientation and coverage of GaHCFe molecules on electrode surface, electron transfer rate constant (k_s) was determined for GaHCFe-CILPE in PBS (pH = 6.4) by measuring ΔE_p at different scan rates. Laviron provided several groups of data corresponding to m^{-1} ($m = RTk_s / nF\nu$, where n, F, R and T have their usual meanings) and $n\Delta E_p$ for those cases that ΔE_p is smaller than 200/n mV and α ranges from 0.3 to 0.7. He also pointed out that the relative error on k_s is at the most about 6% if a relationship for $\alpha = 0.5$ is used. The obtained average value for k_s was 2.0 s^{-1}, assuming $n = 1$ and $\alpha = 0.5$. Also, the results showed that the redox peak current (i_p) intensities increased linearly with the scan rate (ν) in the range between 25 and 500 mV s^{-1}, indicating the presence of a surface-limited redox process. But, at the scan rates in the range between 500

and 2000 mV s^{-1} the plot of i_p versus $v^{1/2}$ was linear, indicating the presences of a diffusion-controlled process which was attributed to the relatively slow diffusion of K^+ into the lattice of GaHCFe.

The effect of pH on the electrochemical behavior of GaHCFe-CILPE was also studied using cyclic voltammetry at different pH values ranging between 2 and 10 and scan rate of 100 mV s^{-1}. The observed redox peak potentials were almost constant and didn't change significantly with increasing pH. At the same time, the observed redox peak current intensities were almost constant and didn't change significantly with increasing pH values up to 6, but then decreased rapidly with further increasing of pH. The decrease of redox peak current intensities in the alkaline solutions is attributed to the slow dissolution of GaHCFe at the electrode surface. So, phosphate buffer solution (PBS, 0.05 M KH_2PO_4 and 0.1 M KCl) with pH of 6.4 was selected as the supporting electrolyte for the subsequent studies.

3.2.1. Electrocatalytic activity of GaHCFe-CILPE towards H_2O_2

To examine the electrocatalytic activity of the proposed sensor towards H_2O_2, cyclic voltammetry was performed using CILPE and GaHCFe-CILPE in PBS (pH = 6.4) at the scan rate of 100 mV s^{-1} in the absence and presence of various concentrations of hydrogen peroxide. As shown in the inset of Fig. 4, the electrochemical reduction and oxidation reactions of H_2O_2 on CILPE are catalyzed at the potentials about +0.2 and +0.6 V vs. Ag|AgCl|KCl_{sat}, respectively similar to that reported previously [17]. On GaHCFe-CILPE (Fig. 4) both i_{pc} and its corresponding i_{pa} increased with increasing the concentration of H_2O_2. Additionally, in the presence of H_2O_2 both i_{pc} and its corresponding i_{pa} increased linearly with $v^{1/2}$, indicating the presences of a diffusion-controlled process which was attributed to the diffusion of H_2O_2. It seems that GaHCFe-CILPE not only catalyzes the reduction of hydrogen peroxide but also is able to catalyze its oxidation reaction.

In order to approve the electrocatalytic oxidation and reduction of H_2O_2 on GaHCFe-CILPE, hydrodynamic voltammetry (stepwise) was performed using GaHCFe-CILPE and CILPE in the presence of hydrogen peroxide (Fig. 5). The anodic current response for H_2O_2 on GaHCFe-CILPE (compared to CILPE) increased slightly when the operating potential was swept from +0.2 to +0.6 V. But, applying potential more positive than +0.6 V (up to +1.0 V) caused a significant increase in anodic current response for H_2O_2 on GaHCFe-CILPE. Also, a significant increase in cathodic current response for H_2O_2 on GaHCFe-CILPE (compared to CILPE) was observed when the operating potential was swept from +0.2 to −0.4 V. The enhancement of anodic and cathodic current responses for H_2O_2 on GaHCFe-CILPE (compared to CILPE) was attributed to the electrocatalytic oxidation and reduction of H_2O_2 on electrode surface, respectively. So, the fabricated GaHCFe-CILPE not only can be used for electrocatalytic reduction of H_2O_2 similar to that reported previously for GaHCFe-CPE [10] but also can be used for electrocatalytic oxidation of H_2O_2. The sensitivity of GaHCFe-CILPE for electrocatalytic oxidation and reduction of H_2O_2 reached to the plateau at the operating potentials about +0.8 and −0.2 V, respectively.

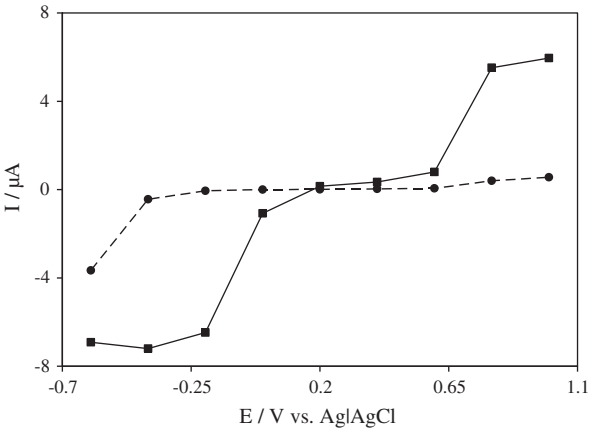

Fig. 5. Hydrodynamic voltammograms obtained for GaHCFe-CILPE (square symbol) and CILPE (circle symbol) in PBS (pH = 6.4) in the presence of 400 μM hydrogen peroxide.

On the basis of previous reports [10,17,19] and the results obtained in this study, the electrocatalytic cycles shown in Eqs. (3) and (4) and in Eqs. (5) and (6) are proposed for the oxidation and reduction of H_2O_2 on GaHCFe-CILPE, respectively.

$$KGa\left[Fe^{(II)}(CN)_6\right] \leftrightharpoons Ga\left(Fe^{(III)}(CN)_6\right] + e^- + K^+ \quad (4)$$

$$2Ga\left(Fe^{(III)}(CN)_6\right] + H_2O_2 + 2K^+ \leftrightharpoons 2KGa\left[Fe^{(II)}(CN)_6\right] + O_2 + 2H^+ \quad (5)$$

$$Ga\left[Fe^{III}(CN)_6\right] + K^+ + e^- \leftrightharpoons KGa\left[Fe^{II}(CN)_6\right] \quad (6)$$

$$2KGa\left[Fe^{II}(CN)_6\right] + H_2O_2 \leftrightharpoons Ga\left[Fe^{III}(CN)_6\right] + 2K^+ + 2OH^- \quad (7)$$

The operating potentials of −0.2 and +0.8 V vs. Ag|AgCl|KCl_{sat}, where the sensitivity of the sensor reached to a plateau, were selected as the optimum working potentials for H_2O_2 detection. Fig. 6 shows hydrodynamic amperometric responses of GaHCFe-CILPE and CILPE

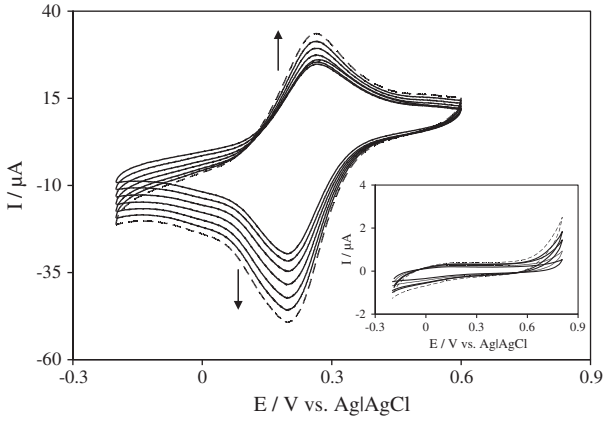

Fig. 4. Cyclic voltammograms of GaHCFe-CILPE and CILPE (inset) in PBS (pH = 6.4) containing different amounts of hydrogen peroxide (0.0 to 2.4 mM with 0.4 mM increment) at the scan rate of 100 mV s^{-1}.

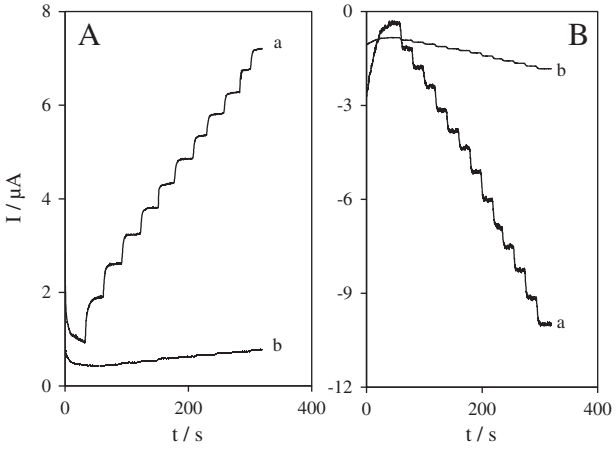

Fig. 6. Hydrodynamic amperometric responses of GaHCFe-CILPE (a) and CILPE (b) in PBS (pH = 6.4) towards successive additions of 40 μM hydrogen peroxide at the operating potential of (A) 0.8 and (B) −0.2 V.

towards the successive additions of hydrogen peroxide solution (40 μM) in PBS (pH = 6.4). As shown in Fig. 6, the addition of H_2O_2 solution causes a sharp increase in the current signal of GaHCFe-CILPE with a response time of less than 6 s which was better than reported for GaHCFe-CPE (<10 s) [10].

At the operating potential of +0.8 V (Fig. 6A), the observed sensitivity for H_2O_2 detection using GaHCFe-CILPE was 13.8 mA M^{-1} and it was 14 times higher than that observed for CILPE (0.96 mA M^{-1}). The sensor, GaHCFe-CILPE, exhibited linear response towards H_2O_2 detection in the concentration range between 5 and 400 μM (r^2 = 0.9922) with a detection limit (S/N = 3) of 1 μM.

At the operating potential of −0.2 V (Fig. 6B), the response of GaHCFe-CILPE was linear towards H_2O_2 in the concentration range between 5 and 500 μM (r^2 = 0.9973). The sensitivity of GaHCFe-CILPE towards H_2O_2 was 18.3 mA M^{-1} and it was 10 times higher than that observed for CILPE (1.9 mA M^{-1}). The detection limit (S/N = 3) and the relative standard deviation (RSD) for 5 times determination of 40 μM H_2O_2 were about 1 μM and 3.9%, respectively. The fabrication reproducibility for six GaHCFe-CILPEs, prepared and used for determination of 40 μM H_2O_2 in different days, was about 9.4%.

It should be noted that working at around 0 mV vs. Ag|AgCl|KCl_{sat} is desirable in practical applications as it decreases non-specific responses to easily oxidizable/reducible interfering compounds common in real samples and also the immobilized protein molecules in electrochemical based biosensors are less susceptible to potential induced degradation.

The effect of pH on sensitivity and operational stability of GaHCFe-CILPE towards H_2O_2 was investigated by hydrodynamic amperometry. Operating potential of −0.2 V vs. Ag|AgCl|KCl_{sat} was applied and amperometric responses of the sensor towards H_2O_2 (400 μM) were recorded in PBS at different pH values ranging between 2 and 10. The sensitivity of the sensor towards hydrogen peroxide was almost constant (about 18 mA M^{-1}) in the pH range between 2 and 6, but then decreased with further increasing of pH values (about 16 and 9 mA M^{-1} at pH of 8 and 10, respectively). The decrease of sensitivity in the alkaline solutions is attributed to the slow dissolution of electrocatalyst, GaHCFe, at the electrode surface. To evaluate the operational stability of the sensor, amperometric responses of the sensor for 10 successive additions of 40 μM H_2O_2 in PBS (pH = 6.4) were recorded every day during one month. The sensor was kept at room temperature in air between days. The response of sensor reduced to 94% of its initial value after 300 (10 × 30 days) assays of 40 μM H_2O_2. The observed operational stability for GaHCFe-CILPE was much better than that reported for GaHCFe-CPE (final peak current reduced to 93% of its initial value after 20 assays of 5 mM H_2O_2) [10].

3.3. Optimizing the fabrication of glucose biosensor

In the "first generation" electrochemical glucose biosensors, the consumption of O_2 or the production of H_2O_2 in the course of enzymatic reaction is electrochemically monitored to determine the concentration of glucose [8,20–22]. Different methods such as entrapment, cross-linking and covalent binding have been proposed for the enzyme immobilization [22–30]. As the surface of the proposed H_2O_2 sensor, GaHCFe-CILPE, does not contain any functionality that readily allows covalent binding of GOx, other strategies must be used for the enzyme immobilization. In this study, GOx was immobilized on the surface of GaHCFe-CILPE using cross-linking with BSA and GLU and entrapment into the Nafion matrix [6].

An aqueous solution containing GOx, GLU and BSA was prepared and used to immobilize GOx on the top of GaHCFe-CILPE. Different amounts of GOx, BSA and GLU were applied during the course of biosensor fabrication. The effect of the loadings of GOx, BSA and GLU on amperometric responses of the biosensor (GaHCFe-CILPE\GOx-BSA-GLU) towards glucose was investigated in PBS (pH = 6.4) at the operating potential of −0.2 V. As shown in Fig. 7A, the amperometric response of the biosensor increases with increasing the amount of GOx, reaches to a maximum at about 1.2 U GOx and then decreases. Clearly, an increase in the enzyme concentration can enhance the biosensor response but a thick enzyme layer can act as a diffusion barrier and reduces the signal intensity. Also, the amperometric response of the biosensor passed over a maximum with increasing the amounts of BSA and GLU. The maximum amperometric response was observed at about 0.8% (w/v) BSA and 0.5% (v/v) GLU. Increasing the amounts of BSA and GLU more than 0.8 and 0.5%, respectively, resulted in an increase in physical instability of the enzyme layer. So, a final volume of 5 μL aqueous solution containing GOx (1.2 U), GLU (0.5% (v/v)) and BSA (0.8 (w/v)) was used for GaHCFe-CILPE\GOx-BSA-GLU fabrication.

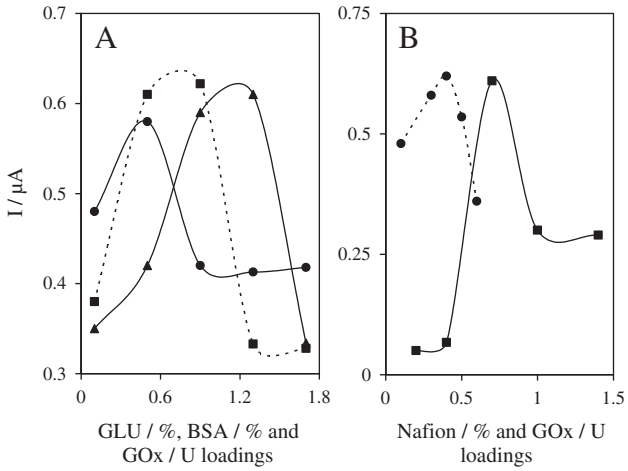

Fig. 7. (A) Influence of GOx (triangle symbol), BSA (square symbol) and GLU (circle symbol) loadings on the hydrodynamic amperometric responses of GaHCFe-CILPE\GOx-BSA-GLU and (B) influence of GOx (circle symbol) and Nafion (square symbol) loadings on the hydrodynamic amperometric responses of GaHCFe-CILPE\GOx-Nafion towards 1.6 mM glucose in PBS (pH = 6.4) at the operating potential of −0.2 V.

Different combinations of GOx and Nafion solutions were also used in order to optimize the immobilization of GOx on top of GaHCFe-CILPE. The effect of the loadings of GOx and Nafion on amperometric responses of the fabricated biosensor was investigated in PBS (pH = 6.4) at the operating potential of −0.2 V. As shown in Fig. 7B, the amperometric responses of the biosensor (GaHCFe-CILPE\GOx-Nafion) passed over a maximum with increasing the amounts of GOx and Nafion. The maximum amperometric response was observed at about 0.4 U GOx and 0.7% (v/v) Nafion. So, a final volume of 5 μL aqueous solution containing GOx (0.4 U) and Nafion (0.7% (v/v)) was used for GaHCFe-CILPE\GOx-Nafion fabrication.

3.3.1. Electroanalytical characteristics of the fabricated glucose biosensors towards glucose

Hydrodynamic amperometric responses of the fabricated glucose biosensors were recorded for a series of standard glucose solutions in PBS (pH = 6.4) at the operating potential of −0.2 V. The response of GaHCFe-CILPE\GOx-BSA-GLU for glucose was linear in the concentration range between 0.1 and 6 mM with a correlation coefficient of 0.9980. The sensitivity and detection limit (S/N = 3) of the biosensor towards glucose were 0.87 mA M^{-1} and 30 μM, respectively. The relative standard deviation (RSD) for repetitive measurements (n = 6) of 1.6 mM glucose was 5.0%. Also, the response of GaHCFe-CILPE\GOx-Nafion for glucose was linear in the concentration range between 0.2 and 6 mM with a correlation coefficient of 0.9987. The sensitivity and the detection limit (S/N = 3) of the biosensor towards glucose were 0.12 mA M^{-1} and 50 μM, respectively. The relative standard deviation (RSD) for repetitive measurements (n = 6) of 1.6 mM glucose was 6.3%. The fabrication reproducibility for six GaHCFe-CILPE\GOx-BSA-GLU and six GaHCFe-CILPE\GOx-Nafion, prepared and used for determination of 1.6 mM glucose in different days was about 6.1 and 7.0%,

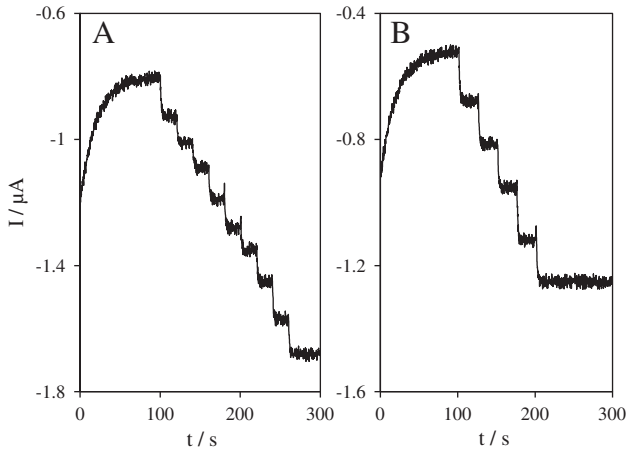

Fig. 8. (A) Hydrodynamic amperometric responses of GaHCFe-CILPE\GOx-BSA-GLU and (B) GaHCFe-CILPE\GOx-Nafion towards successive additions of 1.6 mM glucose in PBS (pH = 6.4) at the operating potential of −0.2 V.

respectively. Fig. 8 shows typical calibration traces recorded for glucose using the fabricated biosensors.

Electroanalytical characteristics of some reported H_2O_2 sensors and glucose biosensors using CuHCFe (copper hexacyanoferrate) and PB modified carbon based electrodes with more or less similar modification strategies are summarized in Table 2 and compared with those obtained in this study using GaHCFe-CILPE, GaHCFe-CILPE\GOx-BSA-GLU and GaHCFe-CILPE\GOx-Nafion and that reported by Yu et al. [10].

Amperometric responses of the fabricated biosensors for 5 successive additions of 1.6 mM glucose were recorded every day during two weeks at the operating potential of −0.2 V to evaluate their operational stabilities. The biosensors were kept at 4 °C in refrigerator between days. It was found that the responses of both biosensors gradually decreased to almost 60% of their initial values after two weeks.

The effect of the presence of potent interfering compounds (12 mM) such as uric acid, ascorbic acid, acetaminophen and dopamine on the amperometric response of both biosensors towards glucose (0.6 mM) was investigated at the operating potential of −0.2 V. The results showed that the additions of acetaminophen, uric acid, dopamine and ascorbic acid, altered the responses of both biosensors for about +5, +7, +9 and −13%, respectively. Direct redox reaction of interfering compounds on biosensor at the applied potential was the main reason for the observed errors.

4. Conclusions

Mixture experimental design and response surface methodology were successfully applied to find the optimal weight ratio of components used for the fabrication of GaHCFe-CILPE. The effect of weight ratios of each component, graphite powder, [C12Py][PF6] and paraffin, on electrode response was clearly shown using mathematical equation models and response trace plot. A pair of well-defined redox peaks due to the redox reaction of GaHCFe with the formal potential and peak potential separation value of +0.216 and +0.073 V was observed for the fabricated electrode. The electron transfer rate constant (k_s) was calculated to be 2.0 s^{-1}, assuming $\alpha = 0.5$. The fabricated electrode exhibited good electrocatalytic activity towards H_2O_2 reduction and oxidation. The proposed sensor was applied for the detection of H_2O_2 at the operating potential of −0.2 V and +0.8 vs. Ag|AgCl|KCl_{sat}. The sensor exhibited high operational stability at pH of 6.4, fast response time (about 6 s), wide linear range, low detection limit and good reproducibility. Additionally, two types of glucose biosensor were fabricated with satisfactory results by the immobilization of glucose oxidase on top of GaHCFe-CILPE using entrapment in Nafion matrix and cross-linking with glutaraldehyde and bovine serum albumin.

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Table 2
Electroanalytical characteristics of some reported H_2O_2 sensors and glucose biosensors using CuHCFe, GaHCFe and PB modified carbon based electrodes.

Electrode	Analyte	E_{app} (mV)	Sensitivity (mA M^{-1})	LDR	DL (µM)	Reference
PBads-CPE	H_2O_2	0.00[b]	30.2	0.5–5000 µM	0.5	[31]
PBelect-CPE	H_2O_2	0.00[b]	1.24	0.25–200 µM	0.25	[31]
GCE/[Bmim][Cl]/PB	H_2O_2	0.00[b]	15.9	5.0–30 mM	n.r.	[32]
ePB/CILE	H_2O_2	−100[a]	11.96	50–6000 µM	1	[33]
cPB-CILE	H_2O_2	−50[b]	86.6	0.01–1 µM	0.01	[6]
cPB-CILE/GOx-BSA-GLU	Glucose	−50[b]	1.9	0.004–2 mM	3	[6]
cPB-CILE/GOx-Nafion	Glucose	−50[b]	0.9	0.001–2 mM	1	[6]
CuHCFe-CILPE	H_2O_2	+800[b]	300	0.2–1000 µM	0.02	[17]
CuHCFe-CILPE/GOx-Nafion	Glucose	+800[b]	100	0.1–1 mM	100	[17]
CuHCFe-CILPE	H_2O_2	−50[b]	12.8	1–1000 µM	0.9	[17]
CuHCFe-CILPE/GOx-Nafion	Glucose	−50[b]	5.6	0.3–2 mM	40	[17]
GaHCF/CPE	H_2O_2	+150[a]	27.9	4.9–400 µM	1	[10]
GaHCFe-CILPE	H_2O_2	+800[b]	13.8	5–400 µM	1	This work
GaHCFe-CILPE	H_2O_2	−200[b]	18.3	5–500 µM	1	This work
GaHCFe-CILPE\GOx-BSA-GLU	Glucose	−200[b]	0.87	0.1–6 mM	30	This work
GaHCFe-CILPE\GOx-Nafion	Glucose	−200[b]	0.12	0.2–6 mM	50	This work

LDR, linear dynamic range; DL, detection limit; n.r., not reported; E_{app}, applied potential; GCE, glassy carbon electrode; CPE, carbon paste electrode; CILE, carbon ionic liquid electrode; CILPE, carbon ionic liquid paste electrode; PB, Prussian blue; PBads-CPE, PB adsorbed CPE; PBelect-CPE, electrodeposited PB on CPE; GCE/[Bmim][Cl]/PB, PB modified with functionalized GCE by imidazolium based ionic liquid; ePB/CILE, electropolymerized PB on CILE; cPB-CILE, chemically PB modified CILE; cPB-CILE/GOx-BSA-GLU, cPB-CILE modified with GOx using crosslinking with bovine serum albumin (BSA) and glutaraldehyde (GLU); cPB-CILE/GOx-Nafion, cPB-CILE modified with GOx using entrapment into the Nafion matrix; CuHCFe-CILPE, CuHCFe modified CILPE; CuHCFe-CILPE/GOx-Nafion, CuHCFe modified with GOx using entrapment into the Nafion matrix; GaHCF/CPE, GaHCFe modified CPE.
[a] vs. SCE.
[b] vs. Ag|AgCl|KCl_{sat}.

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