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Hyphenation of ionic liquid albumin glassy carbon biosensor or protein label-free sensor with differential pulse stripping voltammetry for interaction studies of human serum albumin with fenoprofen enantiomers

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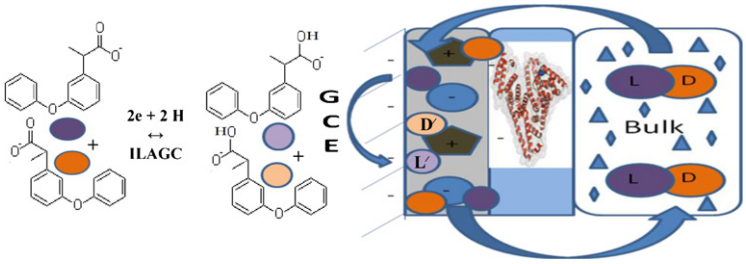
HIGHLIGHTS

- New wearable ionic liquid (IL) biosensor for chiral discrimination of fenoprofen.
- Proving the ability of IL as a mediator and promoter of activity of protein on GCE.
- Simple voltammetric estimation of binding constants of HSA–fenoprofen enantiomers.
- Performing the displacement and reciprocal competitive binding studies on the biosensor.

GRAPHICAL ABSTRACT



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ABSTRACT

A new biosensor or protein label-free sensor composed of 1-butyl-3-methylimidazolium hexafluorophosphates ($BMIMPF_6$)-human serum albumin (HSA) film on glassy carbon electrode (GCE) was produced. Unfortunately, the native proteins themselves are often unstable in physiological conditions. Here, we introduced conjugation with ionic liquid (IL) such as $BMIMPF_6$ which improved the stability and binding affinity of protein onto GCE. A rapid, simple and reliable method for the chiral discrimination and real time protein binding studies of fenoprofen enantiomers with HSA was developed by hyphenating ionic liquid albumin glassy carbon (ILAGC) biosensor with differential pulse cathodic stripping voltammetry under physiological conditions. The electrochemical behavior of chiral fenoprofen was monitored by cyclic voltammetry, from which large response was obtained from L-fenoprofen. The surface coverage of fenoprofen enantiomers was calculated by double potential-step chronocoulometry. The binding constants of chiral fenoprofen with HSA were estimated to be $3.2 \times 10^5 \pm 0.3\,L\,mol^{-1}$ and $0.8 \times 10^4 \pm 0.4\,L\,mol^{-1}$ for L- and D-fenoprofen, respectively giving acceptable precision (SD $\leq$ 0.4) and good agreement with the literature values. The competitive interactions of ibuprofen with fenoprofen enantiomers–HSA were studied giving a significant decreasing in the binding degrees of analytes to HSA. The reciprocal competitive experiments indicated that L-fenoprofen replaced D-fenoprofen from HSA. The proposed electrochemical biosensor holds great potential for chiral discrimination and real time binding studies of drugs with protein.

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1. Introduction

Many diseases by inflammatory processes can progress to a chronic state causing deterioration in the quality of life and a poor prognosis for long-term survival. To address inflammatory diseases effectively, novel therapeutics are required. In actual pharmaceutical research and development, one approach to find and optimize new drug candidates is combinatorial synthesis where a large number of lead structure variations are synthesized in parallel. Consequently, there is an increasing need for testing the extent of biological effectiveness.

Room temperature ionic liquids (RTILs) have taken on a life of their own and now occupy the attention of scientists worldwide. Recent, RTILs are ionic compounds consisting of organic cations and various anions, which are in liquid state at ambient temperature [1]. RTILs were used in the area of electrochemistry and electroanalysis as electrolytes or electrode modifiers for technological applications. RTILs ionic conductivity can be as high as 0.1 S cm^{-1} and bulk electrode material consisting of this liquid acquired this property. Unfortunately for electrochemists, there are only a limited number of RTILs that are really suitable for electrochemistry. In some cases, RTILs that do have sufficient conductivity exhibit meager electrochemical windows and/or show limited chemical stability toward reactive materials. As well, it is necessary to considerer the compensation of the "IR-drop" or "ohmic drop". However, this problem can be circumvented by selecting an appropriate size of working electrode to reduce the specific current [2]. Thus, it is sometimes difficult to identify RTILs having all of the properties necessary to guarantee the success of a specific application.

A major advance in the last few years was the discovery of 1-butyl-3-methylimidazolium hexafluorophosphates ($BMIMPF_6$) that have attracted widespread attention as nonaqueous polar, moderate hydrophobic, nonvolatile, high ionic mobility, chemical and thermal stable [3]. As well, it was found that $BMIMPF_6$ on glassy carbon electrode (GCE) gave a wide electrochemical window (EW) ranged from −2.0 to 2.5 V and the IR-drop was almost negligible (<100 mV) [2]. It was already known that contamination of IL with water shrinks EW [4]. However, it was found that GCE was insensitive to water content in $BMIMPF_6$ [2]. Another major impurity was oxygen which should be eliminated by purging with inert gas during the experiment. Therefore, the appropriate selection of electrode substrate and IL were important to obtain stable liquid deposit in contact with aqueous solution.

Electrochemical methods were used with RTILs to develop new generation of ion selective sensors, voltammetric biosensors and gas biosensors [5]. $BMIMPF_6$ was tested as binder in carbon composite electrode by Safavi et al. [6,7]. Dong et al. mixed room temperature ionic liquids with different carbon materials to form a RTILs-carbon composite material, which was used as modifiers in the studies on the direct electrochemistry of proteins [8]. Moreover, a nano-ZnO and $BMIMPF_6$ composite matrix was fabricated to study the electrochemistry of hemoglobin [9]. Sun et al. also applied different RTILs modified electrodes for the investigation on the electrochemical behaviors of some electroactive substances such as dopamine, ascorbic acid and redox proteins [10–13]. $BMIMPF_6$ was used as both plasticizers and ion exchangers for electrodes with plasticized membranes [14]. Authors of [15] used $BMIMPF_6$ as ionic additive in plasticized membranes of ion-selective electrodes for decreasing of membrane's dielectric constant.

The direct electron transfer between proteins and the electrode surface has received considerable attention recently, because it can be used in fabricating new generation of biosensor devices. Since redox centers are deeply buried within the proteins molecules, the electron transfer between proteins and the electrode is difficult. Therefore, many substrates were used to immobilize proteins on the electrode surfaces. The covalent binding technique was often used for protein immobilization [16,17], but the process of covalent immobilization was somewhat complex and might induce protein denaturation. Recently, the interaction between RTILs and serum albumin (SA) in solution was studied [18]. The optimum simulated results showed that there are two types of binding sites on SA molecules for the binding with $BMIMPF_6$: (1) binding to ionic sites on SA surface driven by hydrogen bonding interaction of anions with amino acid residues and (2) hydrophobic interaction of imidazole ring and the side chain with the hydrophobic cavities of SA molecules. Indeed, RTILs were reported to increase protein stability and activity [19]. Due to the advantages of $BMIMPF_6$ based carbon composite material including large potential window, high ion conductivity and proper interaction with carbon-based electrodes as well as providing mild chemical conditions in preserving the integrity and homogeneity of the protein, we have motivated to fabricate an ionic liquid albumin glassy carbon (ILAGC) biosensor and used it for some electroanalytical applications.

Fenoprofen is nonsteroidal anti-inflammatory, antiarthritic drug that also posses analgesic and antipyretic activities [20]. As far as we know, only one paper dealt with the calculation of fenoprofen–HSA binding constants by HPLC [20]. Voltammetry has excellent features such as less cost, easily automated, rapid analysis and good precision making it in competition with separation techniques. In the present study, a new rapid, simple, sensitive and selective differential pulse stripping voltammetric method coupled with ILAGC biosensor was used for chiral discrimination and precise binding constants of fenoprofen enantiomers with HSA.

2. Experimental

2.1. Apparatus

All voltammetric investigations were performed in a 10.00 mL glass voltammetric cell using commercial available electrode stand (Metrohm, Switzerland). The electrode was connected via IME-663 module (Netherlands). Potentials were controlled using a 3-electrode configuration comprising a rotating ionic liquid albumin glassy carbon disc working electrode (3 mm diameter, Metrohm), a Ag/AgCl (3.00 M KCl) reference electrode and a platinum wire counter electrode. UV–vis measurements were performed by spectrophotometer UV–vis double beam model UVD-3500 (Labomed Inc., USA). The pHs were measured using the Fischer Scientific pH meter model 810 equipped with a combined glass electrode, which was calibrated regularly with buffer solutions (pHs 4.0 and 7.0) at 22 ± 2 °C.

2.2. Chemicals and reagents

Racemic fenoprofen calcium salt hydrate was obtained from Fluka (Germany). Highly purified L-fenoprofen (99.99%) was produced by Western Pharmaceutical Industries, Egypt. Human serum albumin (HSA, ≥99%, Cat. No. A8763) and L-ibuprofen was purchased from Sigma (St. Louis, MO, USA) and was used as received without further purification. Ionic liquid 1-butyl-3-methylimidazolium hexafluorophosphate was synthesized as described elsewhere [21]. Purity of the ionic liquid was checked with an elemental analysis and FTIR. Other chemical reagents were of analytical grade, and double-distilled water was used throughout this study. The 1.00 mg mL^{-1} stock solution of HSA was prepared by dissolving it directly in phosphate buffer. A concentration of 0.067 mol L^{-1} phosphate buffer was used to control the pH at 7.40. $[Fe(CN)_6]^{4-/3-}$ solutions were prepared using 0.067 mol L^{-1} phosphate buffer.

2.3. Preparation of the electrochemical biosensor

The glassy carbon electrode (GCE) was prepared as described in our previous work [22] with little modifications. The electrode was polished at the beginning of the experiments with 0.05 μm aluminum oxide (particle size = 0.10 μm, Metrohm, Switzerland) and was rinsed thoroughly with water to obtain a clean and renewed the electrode surface. The electrode was connected to the potentiostat and placed in the buffer solution; the potential was cycled 50× between −800.00 and +800.00 mV using cyclic voltammetry at a scanning rate 100.00 mV s^{-1}. The cleaned electrodes were dried in a room temperature. The ionic liquid human serum albumin modified GCE was prepared with the following procedure. A 6 μL of the HSA (0.1 mg mL^{-1} dissolved in phosphate buffer at pH 7.4) was mixed with 5 μL $BMIMPF_6$ (3.0 mol L^{-1} dissolved in phosphate buffer at pH 7.4) and then the mixture was dripped on the glassy carbon electrode surface and stayed at room temperature for 8 h or dried by passing very slow rate of hot air for 10 min to form a stable gel-like film. The composite film modified electrode was denoted as $BMIMPF_6$/HSA/GCE (ILAGC) and stored in a refrigerator with fit cap when not in use. The refreshment of the bare glassy carbon surface was achieved by sonicating the modified electrode surface in 0.1 mol L^{-1} hydrochloric acid for 5 min and then acetonitrile for 5 min. The electrode surface was dried and then sensed again with the same composition for further experiments. The fabricated biosensor gave good reproducibility (n = 7, RSD = 4.6%) for the measurement of fenoprofen enantiomers affinity binding with human serum albumin.

2.4. Electrochemical measurements

Into a 10.00 mL volumetric cell, 5.00 μL of 100.00 × 10^{-3} mol L^{-1} L-fenoprofen or rac-fenoprofen was diluted with 67.00 × 10^{-3} mol L^{-1} phosphate buffer, pH 7.4 and mixed homogeneously by rotation for 5 s. Subsequently, the differential pulse or cyclic voltammetric curves were recorded to show the electrochemical changes of the reaction system in the potential range from 0.5 to −0.3 V and all the experiments were performed at room temperature 25 ± 2 °C. Differential pulse experiments were performed at pulse height 50 mV, equilibrium time 10 s and scan rate 10 mV s^{-1}. All the tested solutions were purged with highly purified nitrogen for 10 min before the experiments and a nitrogen environment was kept over the solution during the electrochemical measurements. Attention must be paid such that the working ILAGC biosensor should be removed from the cell solution during the break for the next run in order to have good stable behavior of biosensor. The enantioselective detection was based on the change of the current response $\log(\Delta I_i/\Delta I_{i;\max} - \Delta I_i)$ before and after the modified electrode was immersed in the L- or D-fenoprofen solution [23]. The peak current of the modified electrode was regarded as I_0, and after they were immersed in L- or D-fenoprofen, it was regarded as I_i.

3. Results and discussion

3.1. ILAGC biosensor and fenoprofen enantiomer electrochemical behavior

The standard cyclic voltammograms (CVs) of 5 × 10^{-3} mol L^{-1} $[Fe(CN)_6]^{4-/3-}$ solution (pH 7.4) at a scan rate of 100 mV s^{-1} on bare GCE and ILAGC biosensor were investigated. A standard redox peak could be found for the bare GCE (Fig. 1, curve a), when HSA encapsulated in $BMIMPF_6$ was cast onto the cleaned surface of GCE, it resulted in an obvious decrease of the redox peak (Fig. 1, curve b) in the anodic and cathodic processes, suggesting that the ILAGC

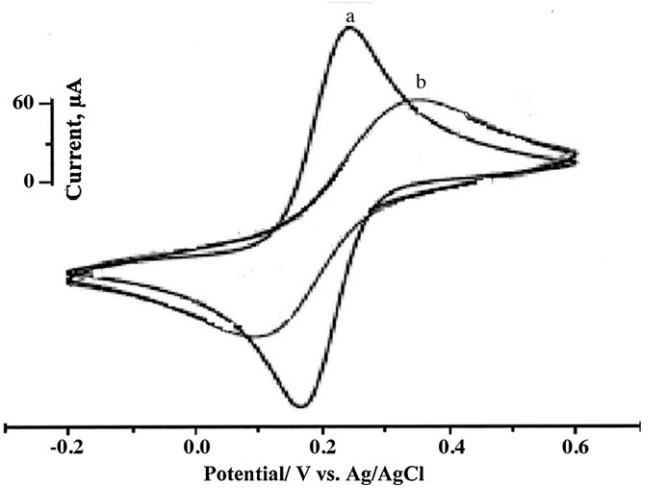


Fig. 1. Cyclic voltammograms of 5 × 10^{-3} mol L^{-1} $[Fe(CN)_6]^{4-/3-}$ solution (pH 7.4) at a scan rate of 100 mV s^{-1} on a bare GCE (a) and ILAGC biosensor (b).

biosensor degraded the redox behavior of the $[Fe(CN)_6]^{4-/3-}$ couple. The conceivable reason was that HSA (pI = 4.7) is a negatively charged protein under physiological conditions which will be balanced by positive charges of cationic IL and consequently plenty of negative anionic IL will be present; thus, the negatively charged redox couple was rejected by the negatively charged sensor surface. However, it is still the diffusion and external stirring driven force quite enough to move redox couple to the sensor surface following by electrochemical reactions. The stability of the proposed biosensor was checked by measuring fresh solution of $[Fe(CN)_6]^{4-/3-}$ under the same conditions for 7 days. A good reproducibility with RSD 5.1% was achieved. As well, the repeatability of the sensing ionic liquid albumin film on a cleaned glassy carbon surface was tested for three times within the same day giving satisfied RSD of 2.4%. Therefore, ILAGC could be considered a stable sensor for electrochemical measurements. This stability could be attributed to the moderate hydrophobicity of IL and its possibility to adsorb on the electrode substrate or attractive interactions with protein as described elsewhere [18]. As well, the duration time of immersing biosensor in the cell solution affected the stability behavior of measurements. Biosensor gave good stability during the voltammetric time scale while its functions were not good enough when it continued in contact with aqueous surrounding electrolyte for more than 8 h. Therefore, it is recommended to remove the working electrode from cell solution during the breaks between measurements.

The electrochemical response before and after the ILAGC biosensor inserted into the L-fenoprofen or rac-fenoprofen solution was investigated by cyclic voltammetry (CV). It was found that the redox current peak decreased after the ILAGC was immersed in 50 μmol L^{-1} of L- or rac-fenoprofen. This behavior could be attributed to that fenoprofen is negatively charged under physiological conditions (pK_a = 4.5) and the movement of such ions to the modified electrode surface may be hindered by electrostatic repulsion. However, the net driven force was enough to move analytes to the sensor surface. It was noticed that the amperometric responses for L- and D-fenoprofen were obviously distinctive, and more interestingly, the ΔI_L was much larger than the ΔI_D. These results indicated that the recognition of L-fenoprofen with the chiral surface is stronger than that of D-fenoprofen. Fig. 2 indicates the repetitive cyclic voltammograms (n = 10) of 5 × 10^{-6} mol L^{-1} rac-fenoprofen after 30 s accumulation time under physiological conditions onto ILAGC biosensor. It was noticed that L- and D-fenoprofen had a quasi-reversible electrochemical behavior with cathodic potentials at 0.15 and 0.07 V and anodic potentials at

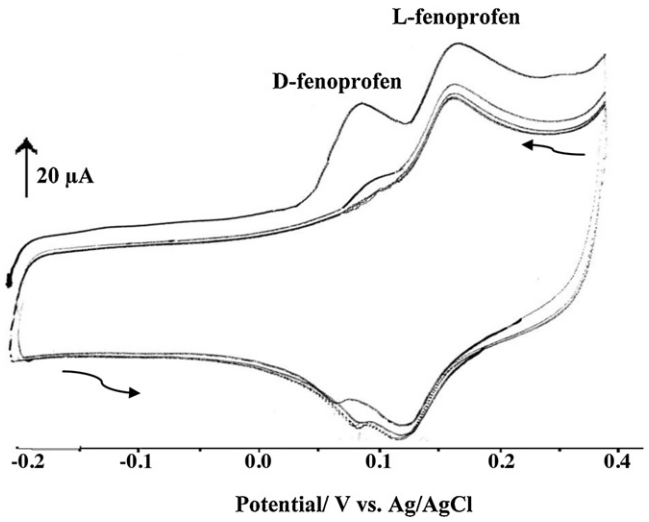


Fig. 2. Repetitive cyclic voltammograms ($n = 10$) of 5×10^{-6} mol L^{-1} of rac-fenoprofen after 30 s accumulation time under physiological conditions on ILAGC biosensor.

0.12 and 0.08 V for L- and D-fenoprofen, respectively. The reductive potential of L-fenoprofen was completely separated from D-fenoprofen ($\Delta V = 80$ mV) while the separation between enantiomers is not good in the anodic polarization ($\Delta V = 40$ mV). Therefore, the electrochemical detector is considered a suitable one to follow the affinity interaction between fenoprofen enantiomers and HSA in the cathodic potential direction. The effect of scan rate on the electrochemical reduction of fenoprofen enantiomers–HSA supramolecular complexation was investigated. It was observed that the peaks currents increased linearly with increasing scan rate (υ) giving a slope value ranged between 0.95 and 0.98 μA s V^{-1} that tended to unity and the correlation coefficient was varied between 0.9991 and 0.9998.

All aforementioned results indicated that larger response signals were obtained from the interaction between HSA and L-fenoprofen compared to the case of D-fenoprofen, with initial good resolution suggesting that HSA could selectively recognize fenoprofen enantiomers. It was reported that the stereoselectivity of HSA is mainly based on the steric, hydrophobic, and hydrogen-bonding interactions between the chiral selector and the target [24], while for fenoprofen enantiomers, the carboxylate groups connect to the chiral carbon atom, possessing different spatial arrangements, could induce a different interacted mode between HSA and fenoprofen enantiomers. So the enantioselective recognition may be due to the different hydrophobic and hydrogen-bonding interactions between HSA and fenoprofen, and stronger binding come from L-fenoprofen compared with D-fenoprofen. That is to say, the difference in the molecular configuration of fenoprofen enantiomers may make degree of matching between HSA and fenoprofen disproportional. This work helps not only to understand the interaction between HSA and fenoprofen, but also to comprehend the enantioselective interaction of proteins and drugs. These electrochemical measurements could be based on the possibility of inclusion of small molecules inside macrocyclic protein/IL phase followed by electrochemical redox process on GC substrate and then analyte molecules excluded into bulk. This suggested electrochemical mechanism (Fig. 3) opened the window for diverse applications of proposed biosensor for the determination of drug enantiomers or so closed similar structure compounds.

Therefore, the current results indicated that ionic liquid $BMIMPF_6$ is considered a suitable mediator and promoter for the electrochemical activity of protein on GCE. This is in an agreement with Wang et al. studies [25] that compared the electrochemical

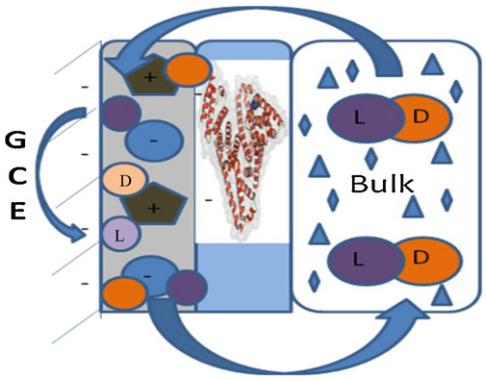


Fig. 3. The proposed separation mechanism of fenoprofen enantiomers on ILAGC biosensor.

behavior of protein (cytochrome complex, Cyt c) in RTILs. The results showed that anions led to a different electrocatalytic property of Cyt c, and attributed to the coordination ability and the acidity of the ILs. Because of increasing the electrochemical activity of Cyt c in $BMIMPF_6$ at neutral conditions, it is expected that $BMIMPF_6$ could make the same behavior with HSA. Therefore, it could be considered that $BMIMPF_6$ enhanced the stability and electrochemical activity of HSA on GCE.

3.2. UV–vis spectra

UV–vis absorption spectroscopy technique was also used to explore the reaction between fenoprofen and HSA. The UV–vis absorption spectra of the different solutions were studied. HSA had a weak absorption peak at about 279 nm due to aromatic amino acids (Trp, Tyr, and Phe) [26]. Fenoprofen owns carboxylate group, which may affect the absorption peak of HSA. The UV–vis spectrum of fenoprofen exhibited a featureless absorption differing from the spectrum of HSA (not shown). The maximum wavelength of racemic fenoprofen with HSA was 276 nm, which showed moderate shifts toward shorter wavelength. This phenomenon confirmed the formation of diastereoisomeric complexes between fenoprofen enantiomers and HSA.

3.3. Differential pulse stripping voltammetric method optimization

3.3.1. The amount of $BMIMPF_6$ + HSA mixture

The influence of amount of the mixture of $BMIMPF_6$ and HSA on the chiral discrimination was investigated in the range of 2–10 μL of $BMIMPF_6$ or HSA in the presence of fixed volume of another component. As shown in Fig. 4, with the amount of HSA increased in the presence of 6 μL, the difference of the peak current for L-fenoprofen was increased at 2–10 μL HSA. As for D-fenoprofen, the difference of the peak current was kept stable at first (up to 6 μL) and then increased much. It is clearly seen that the value of ΔI ($\Delta I = \Delta I_L - \Delta I_D$) was maximum at 6 μL, suggesting that the interaction of HSA and fenoprofen enantiomers had reached saturation, and the continuous increase of ΔI_D with the increased amount of HSA might be due to the physically absorbed amount of D-fenoprofen, thus resulting in the depressed enantioselectivity with bad resolution. In the study, we chose the 6 μL HSA as the optimal amount. The amount of ionic liquid $BMIMPF_6$ was studied in the range of 2–10 μL at constant volume of HSA. The best results were also achieved in the presence of 5 μL of ionic liquid. Therefore, the mixture containing 6 μL of HSA and 5 μL of $BMIMPF_6$ was used as a sensing material onto GCE for the chiral discrimination of fenoprofen enantiomers.

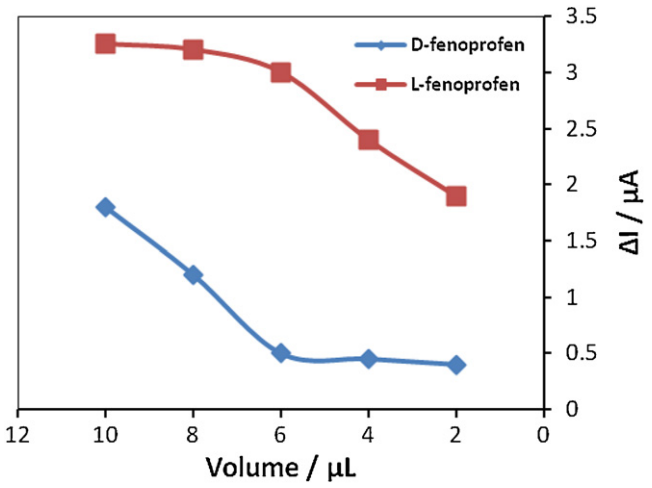


Fig. 4. Effect of the volume of $BMIMPF_6$ + HSA on the optimization of chiral discrimination conditions.

3.3.2. Type of the voltammetric mode

Various instrumental parameters may affect the voltammetric peak potential and its width, so to control and improve the resolution (R); the analyst must know how (R) varies with these parameters. At pH 7.4, the effect of different operational parameters has been tested. Fig. 5 shows a comparison between differential pulse stripping voltammetry (DPSV) curve a, Osteryoung square-wave stripping voltammetry (OSWSV) curve b and linear sweep stripping voltammetry (LSSV) curve c. It was obvious that DPSV and LSSV are more selective than OSWSV for the separation of D- and L-enantiomers. Despite the fact that the enantioresolution for DPSV (R = 1.51) is close to that for LSSV (R = 1.49), DPSV technique was used for further investigations due to its best peak shape and sensitivity. The effect of pulse height, scan rate and equilibrium time on the enantioresolution of rac-fenoprofen was investigated; the optimum values were found to be 50 mV, 10 mV s^{-1} and 10 s,

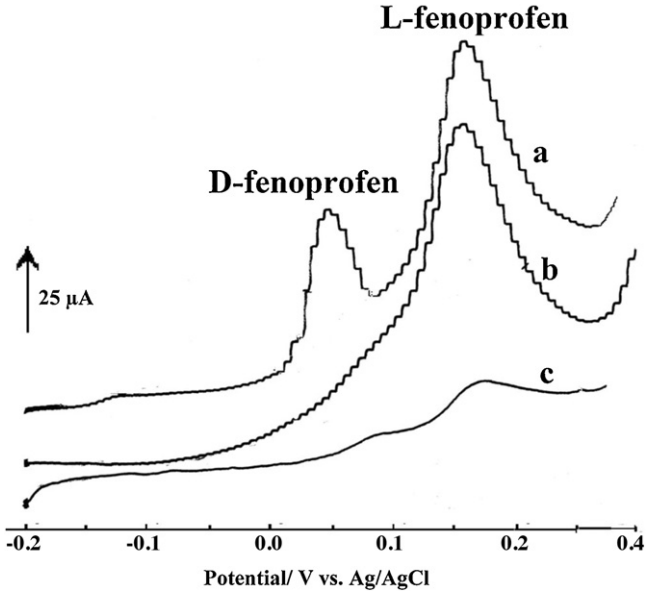
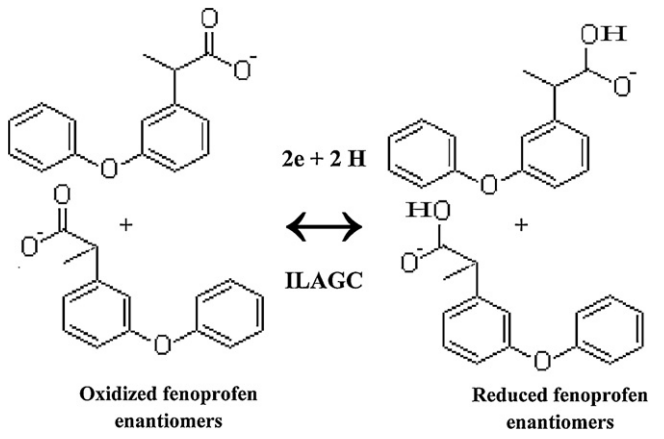


Fig. 5. Comparison between differential pulse stripping voltammetry (DPSV) curve a, Osteryoung square-wave stripping voltammetry (OSWSV) curve b and linear sweep stripping voltammetry (LSSV) curve c on 5×10^{-7} mol L^{-1} of rac-fenoprofen under physiological conditions on ILAGC biosensor.



Scheme 1. The electrochemical scheme of fenoprofen enantiomers on ILAGC biosensor.

respectively. Under these optimal values, the enantioresolution (R) was improved to be 2.85 without distortion of the peaks or the baseline. The electrochemical reduction mechanism of fenoprofen enantiomers on ILAGC could be illustrated as in Scheme 1. It is clear that the electrochemical reduction of carboxyl group close to the chiral center is the main responsible for the separation and voltammetric determination of fenoprofen enantiomers. As well, the presence of negative charge of carboxylate group accelerated the exclusion of enantiomers from protein cavity after electron transfer.

3.3.3. Effect of accumulation time and concentration

Under the foregoing optimal parameters, the preconcentration of rac-fenoprofen at ILAGC biosensor and the application of a subsequent differential pulse scan in the negative direction were tested. It was found that the electrochemical response increased continuously with the interaction times and the ΔI_L was larger than the ΔI_D, indicating that the chiral surface absorbed more L-fenoprofen than D-fenoprofen at the same conditions. This behavior may be attributed to the difference in the strength of complexity between fenoprofen enantiomers and HSA with increasing the number of their molecules captured inside its cavity and/or the possibility to have a competition at the modified electrode surface. Also, the influence of accumulation time on the enantioresolution was studied. The optimal resolution (R = 2.84–2.86) was achieved in the accumulation time range (120–150 s), after that the resolution decreased gradually down to 2.5 at 300 s. This phenomenon might be attributed to the dependence of the equilibriums at the biosensor surface on the amount of fenoprofen species, which was subsequently dependent on the accumulation time. Therefore, 150 s was employed as the best time for chiral fenoprofen discrimination.

The ILAGC biosensor was also used to measure the current response of a series of L-fenoprofen concentration from 10^{-6} to 10^{-8} mol L^{-1} under optimal conditions by DPSV. It was found that larger current gradually increased along with the increasing of L-fenoprofen concentration in a good linear relationship. The linear equation was: conc. (μmol L^{-1}) = 0.052 current (μA) + 0.082 with correlation coefficient (r^2) = 0.9989 and standard deviation (SD) = 0.031. Reproducibility was checked by twenty-one measurements within three consecutive days of 1.00×10^{-7} mol L^{-1} L-fenoprofen under optimal conditions; the relative standard deviation (RSD) of 2.82 was obtained. Detection limit and quantitation limit [27] of 2.52×10^{-9} and 8.10×10^{-9} mol L^{-1} were achieved for L-fenoprofen, respectively. The accuracy of the proposed method was determined by applying the optimized analytical approaches with three spiking replicates at three concentration levels of L-fenoprofen covering the linearity range giving recoveries ranged

from 99.40 to 100.35%. Therefore, the proposed ILAGC sensor could be used for the direct determination of L-fenoprofen in different matrices with good precise data.

Further investigations of the rac-fenoprofen solutions were investigated; the difference of the peak current (ΔI) of the racemic mixture was always between ΔI_L and ΔI_D in the same concentration, and the value of ΔI increased linearly with the increasing concentration, indicating that the racemic D- and L-fenoprofen solution could also be analyzed. All the results confirmed that HSA/IL phase could selectively absorb fenoprofen enantiomers and larger electrochemical response was obtained for L-fenoprofen. As well, the proposed sensor could be used for the determination of enantiomeric purity of fenoprofen in pharmaceuticals.

3.4. Surface coverage of fenoprofen enantiomers on ILAGC biosensor

The charge of the double layer (Q_{dl}) was calculated by applying a double potential-step chronocoulometric technique for the supporting phosphate electrolyte at pH 7.4 and in the presence of rac-fenoprofen (2.82×10^{-7} mol L^{-1}). The adsorbed charge values (Q_{ads}) were calculated for both enantiomers at ILAGC biosensor and found to be 7.97 and 7.75 μC for L-fenoprofen and D-fenoprofen, respectively. The surface coverage could be measured from the division of the number of coulombs transferred by the conversion quantity (nFA) yielding coverage of 5.91×10^{-11} and 5.66×10^{-11} mol cm^{-2} at ILAGC for L-fenoprofen and D-fenoprofen, respectively. The surface coverage values for fenoprofen enantiomers were indicated statistically significant difference at $p = 0.01$. The values of surface coverage confirmed the higher accumulation and inclusion of L-fenoprofen with HSA/IL layer more than D-fenoprofen.

3.5. Determination of the binding constant between fenoprofen enantiomers and HSA

In order to confirm that the interactive effect of HSA to L-fenoprofen was higher than that of HSA to D-fenoprofen, it was important to calculate the binding constant between HSA and D- or L-fenoprofen. The value of binding constant for the interaction of fenoprofen enantiomers with HSA was determined by using Eq. (1) [23,28]; the binding number (m) and binding constant (K) of HSA–m fenoprofen were calculated as follows:

$$\log\left[\frac{\Delta I_i}{\Delta I_{i,\max} - \Delta I_i}\right] = \log\ K + m\ \log[\text{fenoprofen}] \quad (1)$$

where ΔI_i means the change of the peak current of ILAGC biosensor after interacting with D-fenoprofen or L-fenoprofen, respectively; $\Delta I_{i;\max}$ is the maximal peak current change, and [fenoprofen] is the concentration of fenoprofen enantiomer.

Keeping the HSA concentration constant inside the proposed biosensor but changing the concentration of rac-fenoprofen, the plot of log $(\Delta I_i/(\Delta I_{i;\max} - \Delta I_i))$ was obtained with the value of the peak current change. It was clear that the plot of log $(\Delta I_i/(\Delta I_{i;\max} - \Delta I_i))$ against log [fenoprofen] (Fig. 6) results in a straight line both for log [L-fenoprofen] (curve a) and log [D-fenoprofen] (curve b). From the intercept of the linear plot, K_L and K_D were calculated to be 1.58×10^5 and 1.25×10^4 L mol^{-1}, respectively. However, from the slope of the linear plot, $m \approx 1$ for both fenoprofen enantiomers was obtained. Since the value of K_L was larger than that of K_D, it was further evaluated that the binding effect was higher between HSA and L-fenoprofen. Therefore, the observations from the electrochemical methods are reasonable.

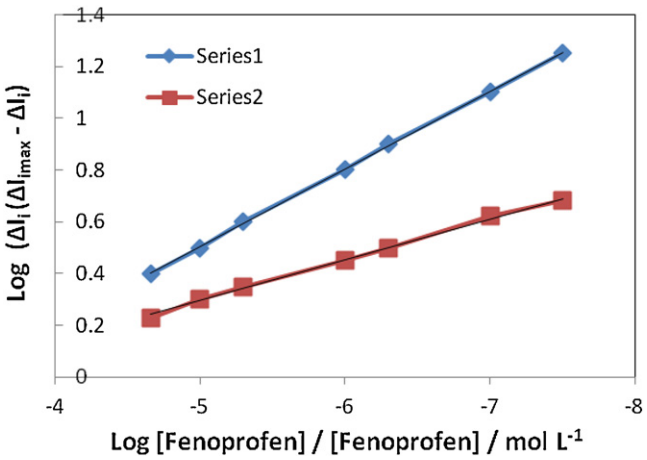


Fig. 6. The plot of log $(\Delta I_i/\Delta I_{i;\max} - \Delta I_i)$ vs. log [fenoprofen]: L-fenoprofen (Series 1) and D-fenoprofen (Series 2).

Table 1
Binding constants (K, L mol^{-1}) of fenoprofen enantiomers with HSA.

Analyte	$K \pm$ SD ($n = 300$) (L mol^{-1})	
	Found value	Literature value
L-Fenoprofen	$3.2 \times 10^5 \pm 0.3$	3.4×10^5 [20]
D-Fenoprofen	$0.8 \times 10^4 \pm 0.4$	1.0×10^4 [20]

The precision of calculated binding constants of fenoprofen enantiomers on ILAGC biosensor was studied for long time analysis ($n = 300$). It was found that the calculated binding constant was $3.2 \times 10^5 \pm 0.3$ L mol^{-1} and $0.8 \times 10^4 \pm 0.4$ L mol^{-1} for L- and D-fenoprofen, respectively (Table 1). When we compared the binding constant value of L-fenoprofen and D-fenoprofen with the literature values, the measured values showed a good agreement with those previous results (Table 1).

3.6. Displacement and competitive binding of L-fenoprofen and D-fenoprofen

The displacement voltammetric studies of L-fenoprofen and D-fenoprofen were studied by increasing the concentration of L-ibuprofen from 1×10^{-8} mol L^{-1} to 2×10^{-7} mol L^{-1} at a constant concentration of 1×10^{-7} mol L^{-1} rac-fenoprofen under physiological conditions. Previous binding study performed [29] indicated that L-ibuprofen had high affinity for HSA on site II (the indole–benzodiazepine binding site). In the present work, it was observed that the increasing of displacer concentration led to high depression in the current response of both enantiomers; e.g. 5×10^{-8} mol L^{-1} L-ibuprofen produced a decrease of about 64.5% and 85.5% of L- and D-fenoprofen, respectively (Table 2) compared to the value in the absence of displacer. Therefore, these results indicated the possibility of direct competitive interaction between L-ibuprofen and L- or D-fenoprofen on HSA-site II.

Table 2
Changes in the current of rac-fenoprofen (1.0×10^{-7} mol L^{-1}) as a result of ibuprofen addition to the supporting electrolyte.

Analyte	Current (μA)	
	Before addition	After addition
L-Fenoprofen	6.67 ± 0.006	2.37 ± 0.003
D-Fenoprofen	4.63 ± 0.005	0.67 ± 0.004

The reciprocal competitive voltammetric studies of L- and D-fenoprofen on HSA were performed by increasing the concentration of L-fenoprofen in the presence of constant concentration of rac-fenoprofen. Results (not shown) indicated the possibility of competitive binding interaction between L- and D-fenoprofen on HSA. Quantitatively, when L-fenoprofen was spiked gradually increased concentration into the system of rac-fenoprofen, the first binding constant of D-fenoprofen–HSA decreased from 0.9×10^4 L mol^{-1} to 3.2×10^3 L mol^{-1}. Therefore, these results confirmed that HSA strongly combined with L-fenoprofen which should replace D-fenoprofen from its binding site (II) in HSA.

4. Conclusions

The present work provides a simple, rapid, sensitive, selective and stable method to discriminate fenoprofen enantiomers by hyphenation of differential pulse stripping cathodic voltammetry with ILAGC biosensor, which has the advantages, such as ease of use and reproducible preparation. All consequences showed that the proposed biosensor exhibited different electrochemical signals for fenoprofen enantiomers where L-fenoprofen gave larger response than D-fenoprofen. The selectivity of proposed method gave a chance to investigate the difference in stability of fenoprofen enantiomers–HSA complexes. This behavior could be attributed to the difference of hydrogen bonds, hydrophobic interactions, and the stereospecific site between HSA and enantiomers. As well, the studied binding of fenoprofen enantiomers with HSA gave acceptable precision and good agreement with the literature values. The displacement studies investigated that competition was occurred in the high-affinity binding site II of HSA. The reciprocal competitive experiments indicated that L-fenoprofen replaced D-fenoprofen from HSA. This work may not only have the potential to discriminate drug enantiomers–HSA interactions but also may bring other ideas in biopharmaceutical field [30]. The proposed electrochemical sensor could enable the use of a single platform for the detection of multiple analytes based on displacement of a charged probe molecule from a surface-bound.

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