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New bioorganometallic ferrocene derivatives as efficient mediators for glucose and ethanol biosensors based on PQQ-dependent dehydrogenases

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Abstract

One known and two new ferrocene-containing mediators incorporating the organometallic moiety and the fragments of natural substrates of oxidative enzymes, viz. 4-ferrocenylphenol (FP), 2-ferrocenyl-4-nitrophenol (FNP), and *N*-(4-hydroxybenzylidene)-4-ferrocenylaniline (HBFA), were studied as electron transfer mediators between the coenzyme pyrroloquinoline quinone (PQQ) of glucose (GDH) and alcohol (ADH) dehydrogenases and the carbon electrode surface. A screen-printed carbon electrode (SPCE) suitable for ADH and GDH immobilization served as a transducer. The electrodes were integrated into a flow-through amperometric cell. All data were obtained at a flow rate of 1 ml min^{-1}. The maximal currents (j_{max}) obtained from the calibration curves for the oxidation of ethanol and D-glucose by ADH and GDH of 2.3 and 3.0 μA, respectively, were obtained when SPCE was modified with HBFA, i.e. with a mediator with a longer arm and a high degree of conjugation. The biosensors were used for ethanol and D-glucose measurements in beverages. There was a good correspondence ($r = 0.978$ for D-glucose and $r = 0.920$ for ethanol) between the data obtained by using the biosensors, on one hand, and by the refractometric or hydrometric methods, on the other. The operational stability of biosensors is determined by the inactivation of the immobilized enzymes rather than by leakage of a mediator from an electrode.

Keywords: Ferrocene; Electron transfer; Pyrroloquinoline quinone; Glucose dehydrogenase; Alcohol dehydrogenase; Screen-printed carbon electrode; Biosensor

1. Introduction

Ferrocene derivatives are widely used mediators (electron shuttles) of the electron transfer between active sites of oxidoreductases and electrodes [1–6]. Amperometric biosensors are often comprised of glucose oxidase and a ferrocene derivative on a carbon paste electrodes [7]. Ferrocenes are slightly soluble in water and this reduces applications of such biosensors. Therefore, biosensors incorporating ferrocene-based redox gels have been introduced [2]. Hale et al. achieved the electrical communication between GO and a conventional carbon paste electrode by using covalently attached ferrocene to insoluble siloxane polymers [5]. Koide and Yokoyama designed an enzyme electrode based on GO and ferrocene-containing polymer made by cross-linking of polyallylamine [8]. Similarly, a reusable pseudo-reagentless glucose biosensor incorpor-

Abbreviations: SPCE, screen-printed carbon electrode; GO, glucose oxidase; HRP, horseradish peroxidase; PQQ, pyrroloquinoline quinone; ADH, PQQ-dependent alcohol dehydrogenase; GDH, PQQ-dependent glucose dehydrogenase; CVA, cyclic voltammetry and cyclic voltammogram.

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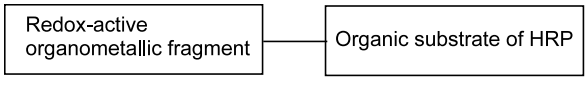
ating a Nafion polymer and 1,1′-dimethylferrocene has also been reported [9]. An HRP electrode with electrochemically deposited ferrocene-modified polyaniline or phenylenediamine film has application in monitoring peroxides in aqueous and non-aqueous media [10,11]. A non-conducting polymer film of 3-aminophenol with immobilized HRP was prepared on a carbon paste electrode by the electrochemical polymerization. This H_2O_2 sensor is applicable for fabricating biosensors for glucose and cholesterol, which are not influenced by easily oxidizable species such as L-ascorbic or uric acid [12]. Mediators chemically bound to a flexible polymer backbone for a better contact with the active centers of GO have also been investigated. This has involved a use of methyl(ferrocenylethyl)siloxane homopolymer and methyl(ferrocenylethyl)dimethylsiloxane copolymer [13]. Tatsuma et al. reported a glucose electrode fabrication using poly(*N*-isopropylacrylamide-co-vinylferrocene) and analyzed its performance both experimentally and theoretically [14]. Recently, some novel 1,1′-dipeptide-ferrocenes were synthesized which are being evaluated for a possibility of their binding to aspartate proteases [15]. Yabuki et al. prepared a polyionic membrane containing physically entrapped microperoxidase and ferrocene on a glassy carbon. Thus made electrode could be used in a matter of 10 days [16].

Some ferrocene derivatives have been tested as mediators in pyrroloquinoline quinone (PQQ) dependent dehydrogenase-catalyzed reactions. Due to the capability of PQQ-dehydrogenases to donate electrons to acceptors other than dioxygen, these enzymes are unique in the biosensor engineering as such biosensors are insensitive to the dioxygen level in samples [17–20]. Promising results have also been obtained using PQQ-dependent membrane-bound aldose dehydrogenase and 1,1′-dimethylferrocene or ferrocene carboxylic acid as mediators [21]. This aldose sensor has been improved by using some new polymer-bound ferrocene derivatives [22]. We have previously shown that 4-ferrocenylphenol adsorbed on graphite electrodes mediates the ADH- and GDH-catalyzed reactions [23]. The biosensors were then improved by using SPCE [24].

The fixation of mediators on various supports is an important component in construction of modern biosensors. Other research is focused on substantial improvement of the mediator performance in terms of stability and reactivity [25]. We have recently introduced the 'biorganometallic' strategy of the mediator design. It is based on combining a redox-active organometallic fragment and an organic moiety reminiscent of the substrate of HRP in one molecule (Scheme 1) [26].



Scheme 1.

Importantly, the mediators made by this approach react fast also with other redox enzymes also including the PQQ-dependent dehydrogenases [23]. This work is a continuation of our studies in this field. It is demonstrated here that 4-ferrocenylphenol (FP) and the new biorganometallic mediators, viz. 2-ferrocenyl-4-nitrophenol (FNP) and *N*-(4-hydroxybenzylidene)-4-ferrocenylaniline (HBFA) are excellent electron transfer mediators between the PQQ coenzyme of glucose and alcohol dehydrogenases on screen-printed carbon electrodes for D-glucose and ethanol monitoring.

2. Experimental

2.1. Chemicals and materials

Carbon rods (Ultra 'F' Purity Ultra® carbon) 3 mm in diameter were obtained from Ultra Carbon Division of Carbon USA Corp. MINICO M 7000 G isolating and Ag MINICO M 4200 pastes were obtained from Emerson&Cuming Specialty Polymers (Westerio, Belgium). A carbon paste electrode on a RAVEN-M carbon black, the latter obtained from Columbian Chemicals Co. (Atlanta, USA), was developed in the Institute of Biochemistry (Vilnius, Lithuania) [27]. Citrate–phosphate buffer (0.1 M, pH 6.0) was used as a default buffer. All inorganic and organic reagents used were of the highest purity available. Doubly distilled water was obtained using a 'Purator Bi' device (Glas Keramic, Germany). 4-Ferrocenylphenol (FP) was obtained as previously described [26].

2.1.1. 2-Ferrocenyl-4-nitrophenol

2-Amino-4-nitrophenol (3.85 g, 0.025 mol), which was obtained from Aldrich, was placed in a 500 ml glass beaker equipped with a stirrer. Water (35 ml) and concd. HCl (6.4 ml) were added. The mixture was cooled to −2 °C using an ice bath and a solution of $NaNO_2$ (2.1 g, 0.03 mmol) in 5 ml H_2O was added in a matter of 40 min to avoid heating the mixture above 0 °C. Excess of nitrite was neutralized by urea and the solution pH was adjusted to 5–6 by NaOAc. After addition of 50 ml water, this solution was mixed using an addition funnel with a stirred solution of ferrocene (3.64 g, 0.025 mol) in 100 ml CH_2Cl_2 placed in a 500 ml round-bottom flask. The mixing continued for 1 h and the temperature of the flask was kept at −3 °C using an ice bath. The temperature was raised to 22 °C; the mixture was stirred for 2 h, and then heated to 60 °C. The color changed from greenish–yellow to dark-red. An aq. layer was separated, $SnSO_4$ was added to reduce the ferricenium ion, and the products were extracted with ether (3 × 30 ml). An organic layer was washed twice with water, combined with the ether solution, and dried with Na_2SO_4. The solvent was removed using a rotary

evaporator, and the residue was column-chromatographed (17 × 3 cm) on Al_2O_3. Unreacted ferrocene (1.9 g) was eluted with petroleum ether and FNP with EtOAc. FNP (1.1 g) was isolated. Crystallization from a 1:1 EtOH–water mixture gave 0.97 g of analytically pure material (33% with respect to consumed ferrocene). M.p. 114 °C. ^{1}H-NMR (δ, $CDCl_3$): 4.32s (5H, Cp), 4.55s and 4.61s (2H, α and β C_5H_4), 7.00d (H5, *J* 8 Hz), 7.96bs (OH), 8.09dd (H4, *J* 8 and 2 Hz), 8.12d (H3, *J* 2 Hz). IR (KBr): 1290, 1336, 3300–3550 cm^{-1}. Anal. Found: C, 59.21; H, 4.17; Fe, 16.93. Calc. for $C_{16}H_{13}FeNO_3$: C, 59.17; H, 4.05; Fe, 17.28%.

2.1.2. N-(4-Hydroxybenzylidene)-4-ferrocenylaniline

4-Ferrocenylaniline (0.4 g, 0.0014 mol) obtained as described elsewhere [28] was dissolved in 10 ml of EtOH on heating and mixed with 4-hydroxybenzaldehyde (0.1 g, 0.0014 mol) in 5 ml of EtOH and the mixture was refluxed for 60 min. The precipitated dark cherry-red material was filtered off, washed with EtOH, and dried using an Abderhalden drying vacuum apparatus. Yield: 66% (0.35 g). M.p. 189–191 °C. IR (Nujol): 3100br (OH), 1635 (C=N), 1600, 1530, 1120, 1000 cm^{-1}. ^{1}H-NMR (δ, d_6-Me_2SO): 4.02s (5H, Cp), 4.34s and 4.78s (2H, α and β C_5H_4), 6.88 and 7.55 (4H, AA′BB′ system from C_6H_4O), 7.17 and 7.78 (4H, AA′BB′ system from C_6H_4N), 8.52s (1H, CH=N), 10.1bs (1H, OH). Anal. Found: C, 72.25; H, 5.18; Fe, 14.46. Calc. for $C_{23}H_{19}NO$: C, 72.46; H, 5.02; Fe, 14.65%.

2.2. Enzymes

ADH was purified from *Gluconobacter* sp. 33 [29] (specific activity 25–40 U mg^{-1}) and used as a solution in 0.02 M potassium phosphate buffer (pH 7.0) containing 0.02% Triton X-100 and 0.5% sucrose. GDH was purified from *Erwinia* sp. 34-1 [30] (specific activity 12 U mg^{-1}) and used as a solution in 0.02 M the same buffer containing 10% glycerol. Invertase (Maxinvert L 1000) of specific activity 72 U ml^{-1} at 20 °C and pH 4.5 was purchased from Gist-Brocades (Germany).

2.3. Construction of screen-printed biosensor and electrochemical flow-through cell

Screen-printed carbon biosensors were constructed by using the following procedure [24]. A Ag track of MINICO M 4200 paste (10 μm thick) on the polyethylene terephthalate film (180 μm) was produced by the screen-printing technology. Printing on the Ag track by the conducting carbon ink [27] produced the surface of the working electrode. Water-insoluble insulating MINICO M 7000 G paste layer was deposited on the electrode surface. Two openings were made, viz. a carbon ink window with an area of 0.04 cm^2 for a working electrode and a 0.5 cm^2 Ag track for an external electric contact. Finally, the working electrode was coated with 3 μL FP, FNP or HBFA (1 mg ml^{-1} in C_3H_6O), 2 μl ADH or GDH, held for 30 min over 25% aq. solution of glutaric aldehyde and covered with semipermeable terylene film [31]. The enzyme electrode was installed into a home-made flow-through three-electrode amperometric cell made from polymethylmethacrylate [24]. The working electrode (0.04 cm^2) is a bottom of the flow cell. A Ag wire (1 mm diameter and 2 cm length) in saturated KCl was used a reference electrode. A Ti rod (2 mm diameter and 2 cm length) was used as a counter electrode.

2.4. Instrumentation

The flow cell was supplied with a ZALIMP 315 peristaltic pump (Poland). The pumping rate was 1 ml min^{-1}. Steady-state currents were recorded using a Radelkis OH-105 polarograph (Hungary). The reference data on D-glucose concentrations in beverages were obtained by using a D-glucose analyzer EKSAN-G (Analita, Lithuania) and a RE 40 refractometer (Mettler-Toledo, Switzerland). Ethanol was quantified by a hydrometer graduated to 0.1 proof. CVA experiments were carried out using an Ag/AgCl reference electrode. All potentials are against this electrode throughout. A Pt coil and a carbon rod sealed in a Teflon tube were counter and working electrodes, respectively. The system was operated by a PA-2 polarographic analyzer (Laboratorni pristroje, Czechoslovakia).

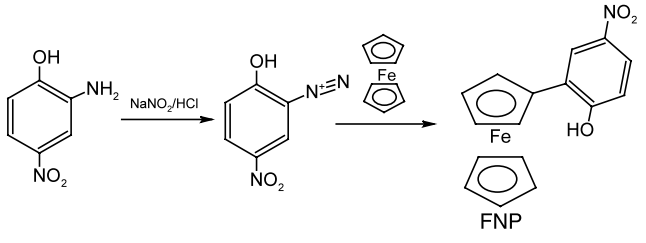
2.5. Determination of D-glucose in beverages

For determining a total amount of glucose and sucrose in soft drinks, sucrose was inverted into D-glucose and D-fructose by Maxinvert L 1000 invertase. A 10 ml sample was diluted 100-fold, incubated for 20 min with 0.1 ml of invertase at 22 °C, and D-glucose was then measured in the flow-through biosensor system.

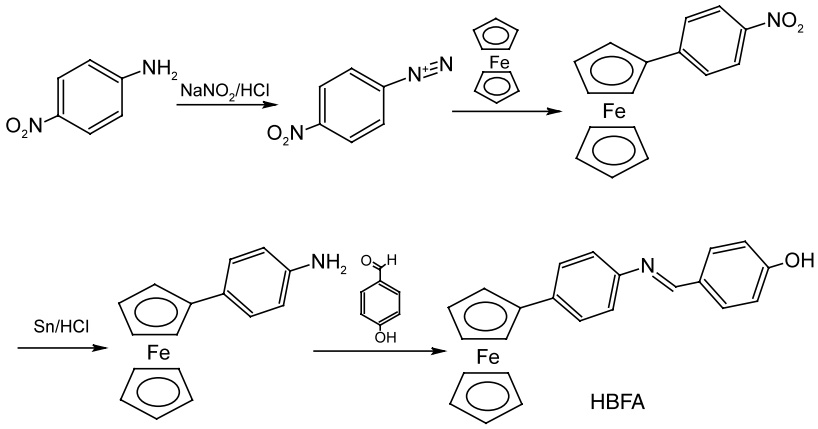
3. Results and discussion

3.1. Synthesis of 'bioorganometallic' mediators

Synthetic routes to new organometallic molecules are shown in Schemes 2 and 3. Preparation of FNP (Scheme



Scheme 2.



Scheme 3.

2) involved transformation of 2-amino-4-nitrophenol into the corresponding diazonium cation which was used for arylation of ferrocene to afford FNP. The Schiff base HBFA (Scheme 3) was made from 4-ferrocenylaniline, the preparation of which was described in detail previously [26], and 4-hydroxybenzaldehyde. Both compounds were thoroughly characterized by spectral and analytical data.

3.2. Electrochemical characterization

FP adsorbed on a carbon electrode was previously studied by CVA [23]. The CVAs exhibited a pair of anodic peaks (E_a) at 0.35 and 0.65 V and poorly resolved cathodic peak (E_c) at 0.2 V. The first anodic peak was ascribed to the oxidation of the ferrocene group and the second referred to the oxidation of the 4-hydroxyphenyl moiety. CVAs of FP, HBFA, and FNP obtained on SPCE are shown in Fig. 1. There is a pair of oxidation peaks corresponding to the oxidation of ferrocene and 4-hydroxyphenyl fragments similar to FP [23]. Slightly lower compared to FP (peak 1 in Fig. 1) value of E_a (0.35 V) was obtained for the oxidation of ferrocenyl moiety of HBFA (peak 4). The phenolic segment of HBFA is oxidized at a more positive potential (0.68 V) as compared with E_a of 0.6 V for FP (cf. peaks 2 and 5). The oxidation potentials of the both moieties of FNP are anodically shifted and seen at 0.47 and 0.75 V (8 and 9). The E_c of 0.35 and 0.5 V characterize the reduction of FP and FNP at the carbon electrode, respectively (3 and 10), whereas two reductive peaks are found for HBFA, viz. at 0.3 and 0.45 V (6 and 7 in Fig. 1). It should be noted that the peak current for the oxidation of phenolic moieties of ferrocene derivatives decreases with increasing the number of scans. This effect was previously studied by the example of FP and accounted for electrochemical polymerization of the 4-hydroxyphenyl fragments [23]. This process also decreases slightly the peak current from the ferrocenyl groups. At lower potentials, when no polymerization occurs, all investigated mediators display steady redox behavior of the ferrocene moiety (Fig. 2). In addition, the values of E_a and E_c are practically independent, but the peak currents increase proportionally to the sweep rate. This emphasizes good electrochemical reversibility of the ferrocenes and suggests that they could be promising mediators in the ADH and GDH-catalyzed

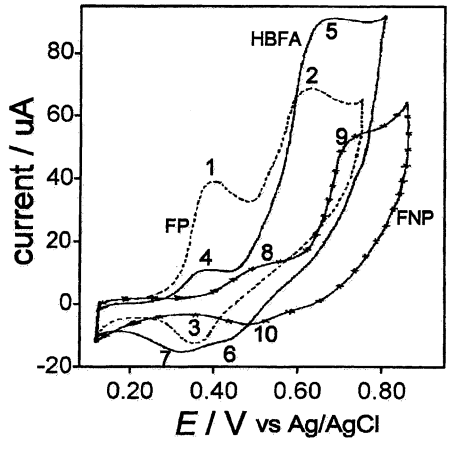


Fig. 1. CVAs of FP, FNP, and HBFA adsorbed on SPCE: 0.1 M, KCl; scan rate, 10 mV s^{-1}. See text for details.

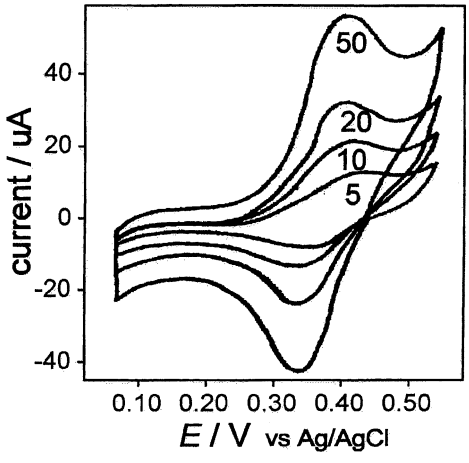


Fig. 2. CVAs of HBFA adsorbed on SPCE at different potential sweep rates (in mV s^{-1}).

ethanol and D-glucose oxidations at potentials, when the polymerization of the 4-hydroxyphenyl moieties is eliminated.

3.3. Evaluation of mediating efficiency of FP, HBFA, and FNP in glucose biosensor

The main objective of this work was evaluating ferrocene compounds as effective electron transfer mediators in GDH-catalyzed oxidation of D-glucose, since GDH show no electrocatalytic behavior on carbon electrodes in the absence of mediating material. The current responses of the carbon electrode modified with GDH, on one hand, and FP, HBFA, or FNP, on the other, were investigated as a function of the working potential. It was always below 0.5 V, thus eliminating the electropolymerization. Measurements were performed in a flow system in the buffer using 0.5 mM glucose solutions. CVAs obtained are shown in Fig. 3. The highest catalytic current, close to the FP-modified electrode, was observed for HBFA as a mediator. The maximal responses for FP and FNP electrodes are obtained at potentials around 0.4 V, i.e. slightly higher than required for the highest response of the HBFA-modified electrode. Obviously, a highly conjugated arm of HBFA facilitates the electron exchange rate. This leads to increased sensitivity of the biosensor even at low potentials (Fig. 3). The negative mesomeric effect of the nitro group in FNP shifts the peak anodically and decreases the rate of the electron exchange between the active center of enzyme and the mediator.

Fig. 4 shows the glucose calibration curves for GDH electrodes modified with FP, HBFA, and FNP at the working potential of 0.4 V, which was selected for comparing ferrocenes used. The highest current density (28 μA $(cm^2\ mM)^{-1}$) was observed for the HBFA-modified carbon electrode. For FP and FNP this equals 10.5 and 1.8 μA $(cm^2\ mM)^{-1}$, respectively. The apparent Michaelis constants K_M^{app} for glucose calculated from data in Fig. 4 equal 2.4, 0.83, and 2.7 mM for HBFA, FP, and FNP, respectively. Clearly, structurally different ferrocenes display different mediating abilities. The best performance for HBFA, viz. a compound with a 4-iminomethylphenolic arm, is likely due to the fact that the latter is longer than the phenolic arms of FP and FNP. An extra reason for the better response could be higher hydrophobicity of HBFA that enables a better interaction between ferrocene and the cofactor buried in the protein globule of the enzyme. A clear dependence of the current response on the polymer structure was also observed for PQQ-dependent aldose dehydrogenase by Smolander et al. [22].

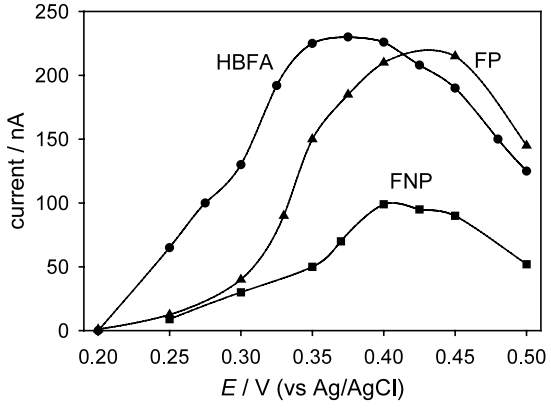


Fig. 3. Hydrodynamic voltammograms for the GDH and FP, HBFA, FNP-modified carbon electrodes. Conditions: flow rate, 1 ml min^{-1}; 22±2 °C, 50 mM acetate (pH 6), 1 mM Ca^{2+}.

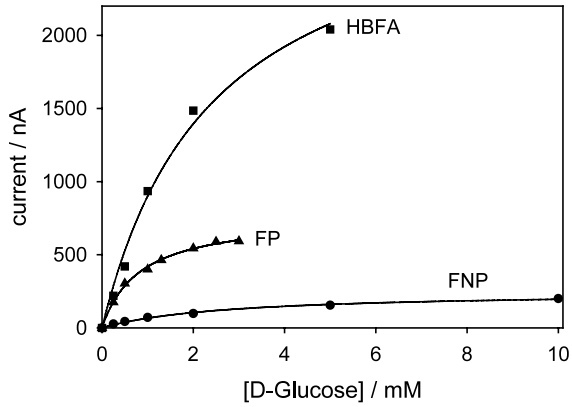


Fig. 4. Calibration curves for D-glucose obtained using the carbon electrode modified with GDH and HBFA, FP, and FNP. Applied potential, 0.4 V; carrier buffer, 50 mM acetate (pH 6) with 1 mM Ca^{2+}.

3.4. Evaluation of mediating efficiency of FP, HBFA, and FNP in ethanol biosensor

In contrast to GDH, ADH adsorbed on carbon electrodes is involved in non-mediated electron exchange with an electrode in the presence of ethanol [32,33]. This is due to the structure of the enzyme. ADH contains four heme moieties, which act as bridges for the electron transfer from PQQ to the electrode surface. Therefore, the catalytic efficacy of the mediated oxidation of ethanol was compared with the non-mediated process. The current responses of the carbon electrode modified with ADH and FP, HBFA, FNP or ADH only were obtained as a function of the working potential. Measurements were performed in a flow system in the buffer using 1 mM ethanol solutions. CVAs obtained are shown in Fig. 5. The highest and the lowest catalytic currents were observed for HBFA as a mediator and at a bare carbon electrode, respectively. The relative mediator efficacy (HBFA > FP > FNP) was as in the GDH case, whereas a difference in the peak positions was smaller. This could be accounted for in terms of the cooperative effect of the PQQ and heme groups. As a

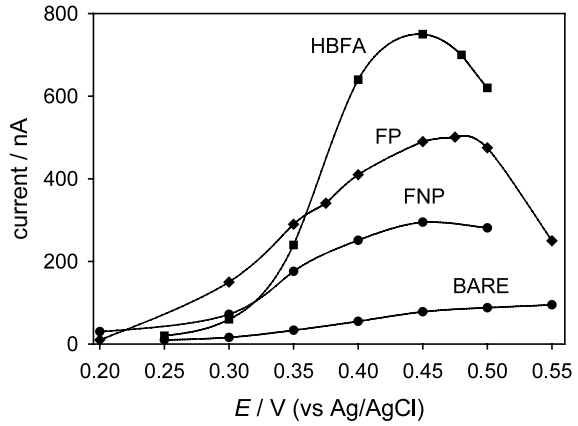


Fig. 5. Current response of the bare ADH-modified carbon electrode and same electrode additionally modified with ferrocene derivatives HBFA, FP, and FNP. Data is obtained in a flow system; 0.5 mM EtOH; carrier buffer, 50 mM acetate (pH 6) with 1 mM Ca^{2+}.

result, the catalytic current is comparable for all three mediators investigated.

The ethanol calibration curves obtained using the ADH carbon electrode and ADH electrodes modified with FP, HBFA, and FNP are shown Fig. 6. The highest current density (28 μA $(cm^2\ mM)^{-1}$) was reached for the HBFA-modified electrode. It is at least 10 times higher compared to the current obtained at the ADH-modified bare carbon electrode. For the FP- or FNP-modified electrodes, the values are 17.5 and 12 μA $(cm^2\ mM)^{-1}$, respectively. Thus, HBFA provides the highest electrocatalytic activity for the both PQQ-dependent enzymes. One and the same catalytic mechanism is obviously operative in both cases (Section 3.3). The values of K_M^{app} calculated from data in Fig. 6 for HBFA, FP, and FNP equal 1.0, 1.5 and 2.5 mM, respectively. The numbers are slightly higher than the Michaelis–Menten constants obtained for the ADH reactions in solution [29].

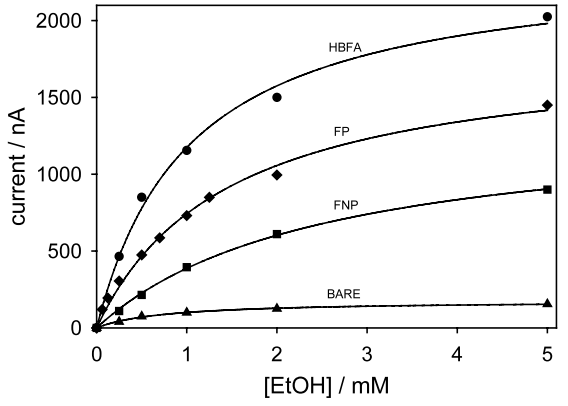


Fig. 6. Calibration curves for ethanol obtained on the bare ADH-modified carbon electrode and same electrode additionally modified with ferrocene derivatives HBFA, FP, and FNP. Data is obtained in a flow system; 0.5 mM EtOH; carrier buffer, 50 mM acetate (pH 6) with 1 mM Ca^{2+}.

3.5. Kinetic evaluation of stability of biosensors

The operational stability of the GDH- or ADH-modified carbon electrode with adsorbed HBFA was studied in a flow system. The performance of the both biosensors was investigated in a matter of 3 weeks at room temperature at pH 6. Measurements were made using standard ethanol or D-glucose solutions (0.5 mM) at 0.4 V. Different kinetics of functioning of HBFA and ADH- or GDH-modified carbon electrodes (Fig. 7) indicates that the stability of the immobilized enzyme plays a dominant role. This was proven by adding a soluble mediator, i.e. phenazine methosulfate (data not shown). The response of the ethanol biosensor decreases by ca. 50% in 2 days presumably due to dissociation of poorly bound ADH molecules from the carbon surface. However, a decrease in the sensor response is indistinct after 2 weeks. In contrast, the response of the glucose biosensor increases during first working day. The protein molecules are likely tightly bound to the electrode surface and both GDH and the mediator adopt an arrangement most favorable for the mutual contact. Studies of time-dependencies of the performance of both biosensors suggest that it is unaffected by the mediator leaking, but the immobilization procedure based on the cross-linking with glutaric aldehyde is more appropriate for GDH than for ADH.

3.6. Determination of D-glucose and ethanol in beverages by carbon electrodes modified with FP and ADH or GDH

The biosensors inserted in a flow system were evaluated for measuring D-glucose and ethanol in various non-alcoholic and alcoholic beverages. Soft drinks produced in Lithuania (with or without CO_2) are made of oranges, peaches, pears, grapes or other fruits. They contain 0.1–200 mM of D-glucose, which

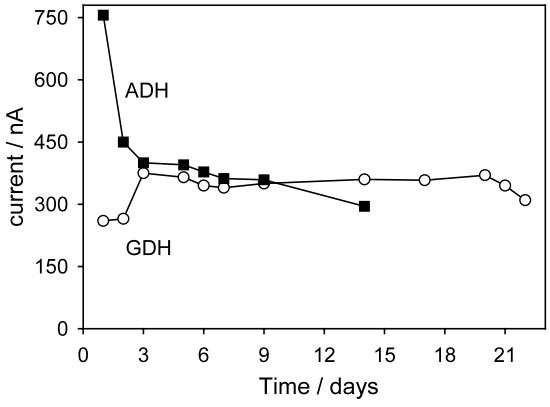


Fig. 7. Stability of ethanol and glucose biosensors assembled on the ADH- and GDH-modified carbon electrodes using FP as a mediator. Experimental details: working potential, 0.4 V; carrier buffer, 50 mM acetate (pH 6) with 1 mM Ca^{2+}; response to 0.5 mM substrate, at least 15 assays per day.

was quantified by using the carbon electrode modified with GDH and FP. Samples were analyzed after dilution and the invertase-catalyzed conversion of sucrose into D-glucose. The data obtained by using the biosensors, an EKSAN-G analyzer, and refractometrically are compared in Fig. 8. The correlation coefficients of 0.978 and 0.975 as well as insignificant intercepts are indicative of low background interfering currents. Therefore, impurities and electrochemically active compounds in beverages have a low influence on the biosensors response.

The ADH/FP-based sensor was evaluated by measuring ethanol in red and white wines that contain 9–18 wt.% ethanol. Samples were analyzed in a flow-through system after ca. 10 000-fold dilution with the buffer. Comparison of the bioanalytical and the standard hydrometer-based methods is presented in Fig. 9. The correlation coefficient equals 0.9204 indicating a good applicability of the biosensor for measuring D-glucose and ethanol levels in foodstuffs.

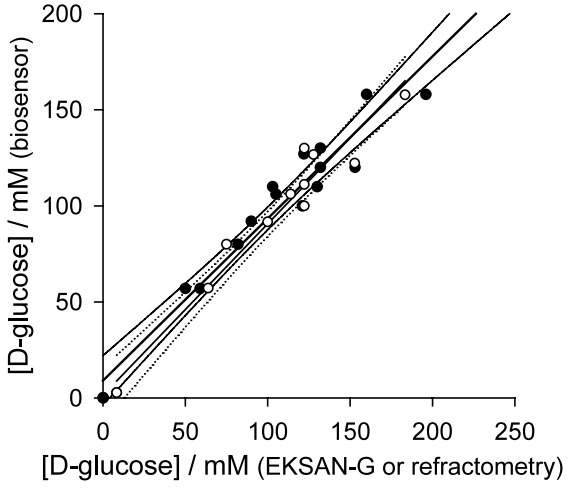


Fig. 8. Comparison of the data obtained using the glucose biosensor and refractometer (○) or analyzer 'EKSAN-G' (●). See text for details.

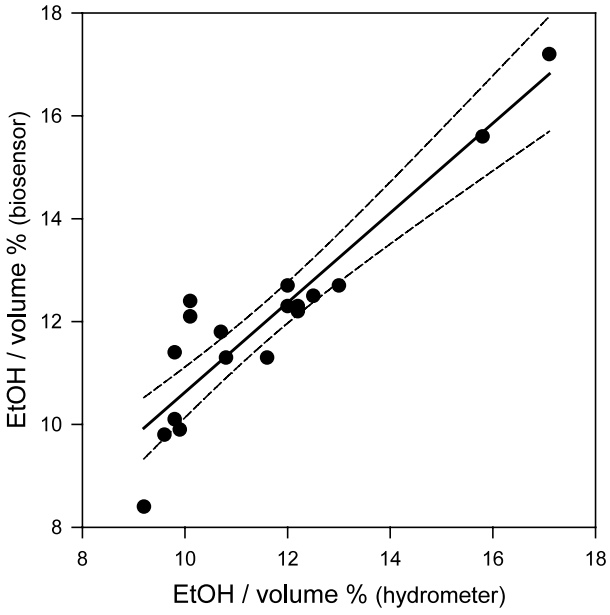


Fig. 9. Comparison of the data obtained using the ethanol biosensor and hydrometer. See text for details.

4. Conclusions

The carbon electrode modified with 'bioorganometallic' ferrocene derivatives permits the detection of ethanol and D-glucose in the potential range 0.25–0.45 V. The maximal sensitivity of biosensors for ethanol and D-glucose were reached by using a carbon electrode modified with HBFA and the corresponding PQQ-dependent enzymes. The biosensors thus prepared were used for measuring ethanol and D-glucose in beverages. Good agreement between the data obtained bioanalytically, on one hand, and refractometrically or hydrometrically, on the other, demonstrates the applicability of the biosensors for quantifying ethanol and D-glucose in beverages.

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