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Evaluation of redox mediators for amperometric biosensors: Ru-complex modified carbon-paste/enzyme electrodes

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Abstract

The properties of reagentless amperometric biosensors are mainly governed by the interaction of the used redox enzyme and the redox mediators used to facilitate the electron-transfer reaction. Both the used redox mediators and the redox enzymes differ concerning their hydrophilicity and their properties within the matrix of a carbon-paste electrode. Since there is no general procedure which is applicable for any enzyme in combination with any redox mediator, optimisation is necessary for each possible combination.

Three approaches for the development of biosensors were investigated using carbon-paste electrodes enriched with redox mediator as a base in all sensor architectures. A class of redox mediators with the common formula $Ru(LL)_2(X)_2$ (where LL are 1,10-phenantroline or 2,2′-bipyridine type ligands, and X is an acido ligand) was investigated. In the first approach, enzymes were integrated into the carbon paste; in the second, the enzymes were adsorbed on the surface of the mediator-containing carbon-paste electrode and held in place by a Nafion film; and in the third approach, enzymes were entrapped in polymer films, which were electrochemically deposited onto the electrode's surface.

The properties of the obtained biosensors strongly depend on the sensor architecture and the specific features of the used enzyme. Thus, our investigation using three different sensor architectures can provide valuable information about the possible interaction between a specific enzyme and a redox mediators with specific properties.

Keywords: Ru-complex; Biosensor; Electron transfer; Carbon-paste electrode; Redox mediator

1. Introduction

Electron-transfer processes between the active site of an (immobilized) enzyme and the electrode surface play an important role in determining how an amperometric biosensor effectively carries out its function of measuring substrate-dependent currents [1,2]. Due to the often encountered insulation of the enzyme-integrated active site by protein shell, direct electron transfer becomes difficult. Thus, alternative electron-transfer pathways, e.g. via free-diffusing low-molecular weight redox-active molecules, have been demonstrated [3].

Consequently, a vast amount of different artificial redox mediators was described in the last two decades and combined with a variety of different redox enzymes in biosensors. The most important features which are essential for the function of redox mediators are (i) a fast electron-transfer rate (K_{ET}) with both the active site of the enzyme and the electrode surface, (ii) a low redox potential in order to avoid co-oxidation or co-reduction of interfering compounds, (iii) sufficient chemical stability of the oxidized and the reduced form, and (iv) a low reorganization energy [4] to allow fast electron transfer even over significant electron-transfer distances.

Usually, the possibility of an interaction between a specific enzyme and a redox mediator is investigated by means of voltammetry of the dissolved enzyme in the presence of the mediator and saturation concentrations of the enzyme's substrate. In the absence of the substrate, only the redox wave of the mediator is observed, while in the presence of the substrate a typical electrocatalytic cyclic voltammogram is recorded from which the electron-transfer rate between enzyme and redox mediator can be deduced

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[5]. However, even if small electrochemical cells are used, the loss of the often valuable mediator compounds and, concomitantly, the loss of the enzyme do not allow a thorough investigation of a specific redox mediator with a number of enzymes, using a variety of different electrolyte solutions, pH value, etc. [6].

Carbon-paste electrodes are frequently applied in amperometric biosensors [7–10]. Usually, a suitable redox mediator is mixed in the carbon paste and the enzyme is either also integrated in the carbon paste or it is immobilized at the surface of the carbon-paste electrode. Although the overall sensing mechanism is still under discussion, it can be assumed that the carbon-paste integrated redox mediator is partially solubilized in the interface between carbon paste and electrolyte where it may freely diffuse between conducting carbon particles and the active site of the enzyme. Most of the presently applied redox mediators suffer from low stability, slow electron-transfer rate especially with some enzymes, or poor solubility in aqueous electrolyte which is indispensable for the preservation of enzymatic activity. Transition-metal complexes have been proved to be suitable redox mediators with a variety of enzymes. They offer a certain adaptability due to the possibility of varying the ligand shell of the central metal [11] and hence modulating the redox potential, the charge, and the interaction potential with a specific enzyme [12].

Recently, Os- and Ru-complexes have been evaluated with respect to their ability to act as redox mediators with different oxidoreductases. Ru-complexes of the type $[Ru(LL)_2X_2]$, where LL is 1,2-bypiridine or 1,10-phenantroline and X is an acido ligand, were found to undergo rapid interaction with some oxidoreductases, such as FAD-dependent glucose oxidase (GOx), and horseradish peroxidase (HRP) [13]. In addition, these mediators could be bound to the surface of redox proteins by means of a ligand exchange reaction with the protein-bound histidine residues [14,15]. The obtained mediator-modified enzymes showed improved electron-transfer rates compared with the native enzyme [16,17].

In this communication, a variety of $[Ru(LL)_2X_2]$ complexes, where LL equals 1,10-phenantroline or 4,4′-substituted-2,2′-bipyridine ligands and X is an acido ligand, were investigated with respect to their properties in amperometric biosensors using different enzymes and three different types of carbon-paste electrode architecture. Besides the redox potential, the mediators significantly differ concerning their solubility in aqueous solution and within the matrix of the carbon paste. Since also the investigated enzymes differ with respect of their hydrophilicity, stability within the carbon paste, and the possible interaction between the enzyme and the redox mediator, no general procedure is applicable for the optimisation of combinations of redox mediators and enzymes. Thus, our investigation using three different sensor architectures can provide valuable information about the possible interaction between a specific enzyme and redox mediators with specific properties. In this respect, the proposed sensor architectures allow for a rapid screening of mediator properties in combination with different types of enzymes.

2. Experimental

2.1. Chemical and enzymes

Quinohemoprotein alcohol dehydrogenase (QH-ADH) from *Gluconobacter* sp. 33 and pyrroloquinolinequinone-dependent glucose dehydrogenase (PQQ-GDH) from *Erwinia* sp. were kindly provided by Prof. Valdas Laurinavicius, Insitute of Biochemistry, Vilnius, Lithuania. The QH-ADH enzyme was prepared following a procedure described previously [18]. The enzyme preparation had a specific activity of 60 units ml^{-1} (in 0.02 mM phosphate buffer, pH 7.0, containing 0.02% Triton X-100 and 0.5% sucrose). PQQ-GDH was obtained as previously described [19], and the enzyme with a specific activity of 55 units ml^{-1} was dissolved in 0.02 M phosphate buffer (pH 7.0) containing 10% glycerol. NAD^+-dependent glucose dehydrogenase (NAD-GDH) from calf liver (220 units mg^{-1}) and NAD^+-dependent glycerol dehydrogenase (NAD-GlyDH) from *Celluolomonas* sp. (69 units mg^{-1}) were purchased from Sigma (Steinheim, Germany). NAD^+-dependent alcohol dehydrogenase (NAD-ADH) from *yeast* was obtained from Roche Diagnostics (Mannheim, Germany). For electrode preparation 1 mg of the NAD^+-dependent dehydrogenase was dissolved in phosphate buffer.

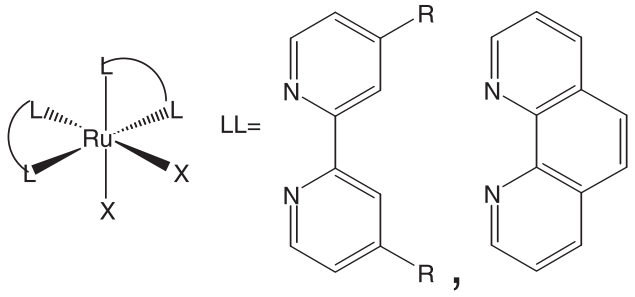
D-Glucose was from Merck (Darmstadt, Germany), glycerol was from Sigma, ethanol and KH_2PO_4 were purchased from Baker (Deventer, Netherlands). $Na_2HPO_4{*}7H_2O$ was from Riedl-de Haen (Seelze, Germany). NAD^+ was obtained from Biomol Feinchemikalien (Hamburg, Germany). Silicon oil was purchased from Bayer (Leverkusen, Germany). Carbon powder was obtained from Ringsdorf Kohlenstoffwerke, (Godesberg, Mehlem).

D-Glucose solution was prepared by dissolving 3.6 g of D-glucose in 5 ml of phosphate buffer (50 mM). NAD^+ solution was prepared by dissolving 179.2 mg of NAD^+ in 500 μl of phosphate buffer (50 mM). The Ru-complexes used (Scheme 1) were kindly provided by Dr. A.Yu. Ershov, St. Petersburg, Russia, and had been synthesized as described previously [20].

Anodic electrophoretic deposition paint (EDP, Elektrodepositionslack Glassophor ZQ 8-43225-Canguard) was obtained from BASF Farben und Lacke (Münster, Germany). For more detailed information on the content of these paints and the preparation of the related biosensors, see Refs. [21–23].

2.2. Preparation of carbon-paste electrodes

Carbon paste was prepared by thorough mixing of 400 μl of silicon oil with 1100 mg of carbon powder in a mortar.



	LL	X	R
1	Bpy	Cl^-	H
2	Bpy	SCN^-	H
3	Bpy	Cl^-	Me
4	Phen	CO_3^{2-}	H
5	Phen	Cl^-	H

Scheme 1.

The obtained carbon paste was used as the basis for the preparation of different Ru-complex modified carbon pastes.

Five milligrams of each Ru-complex (see Scheme 1) was dissolved in 500 μl of ethanol; the resulting solution was added to 50 mg of the base carbon paste, and the paste was intensively mixed to obtain a homogeneous distribution of the mediator within the carbon paste. Ethanol was allowed to evaporate and the "dried" paste was densely packed into a yellow "Eppendorf" tip (used for 20–200-μl pipettes). After pressing the carbon paste into the conically shaped plastic tip, the tip was cut off approximately 2 mm from the narrow end. A copper wire was inserted through the opposite wide end in order to establish electrical contact.

2.3. Preparation of carbon-paste based biosensors

"A" type biosensors: 5 mg of corresponding enzyme and 55 mg of the Ru-complex enriched carbon paste were mixed, and the enzyme- and mediator-containing carbon paste was filled into the plastic tip as described above. In the case of QH-ADH and PQQ-GDH (these enzymes are only available dissolved in buffer), 30 μl of the enzyme preparation was mixed with the carbon paste. The water was allowed to evaporate, yielding an enzyme-modified carbon paste which was handled in a similar manner as the pastes obtained with lyophilized enzymes.

"B" type biosensors: 5 μl of a freshly prepared enzyme solution was placed on the surface of carbon-paste electrode and allowed to dry at room temperature (23 °C). Afterwards, 3 μl of 5% Nafion solution in ethanol was dropped on the carbon-paste electrode surface with the adsorbed enzyme. After drying for about 30 min, a Nafion membrane was formed which entrapped the previously adsorbed enzyme on the surface of the mediator-modified carbon-paste.

"C" type biosensors: Mediator-modified carbon-paste electrodes were intensively rinsed with water. One-hundred microliters of the enzyme solution and 100 μl of the "Canguard" polymer suspension were mixed and stored for at least 30 min in the refrigerator. The carbon-paste electrode was then immersed together with a reference electrode and a Pt-wire counter electrode into the cold enzyme/polymer solution. A potential pulse profile (2600 mV for 1 s; 800 mV for 1 s and 0 mV for 5 s; vs. Ag/AgCl) was applied 10 times, which led to the growth of an enzyme-containing polymer hydrogel on the surface of the carbon-paste electrode. The obtained biosensors were rinsed thoroughly with water and 0.1 M phosphate buffer (pH 7).

2.4. Electrochemical measurements

Constant-potential amperometry was performed using a Biometra PED 300 bipotentiostat (Biometra, Göttingen, Germany) in a conventional three-electrode configuration. Pt-wire and Ag/AgCl/3 M KCl electrodes were used as auxiliary and reference electrodes, respectively. The current-over-time curve was recorded using serial communication with a PC and an in-house developed software. Cyclic voltammetry was performed using an EG&G Princeton Applied Research Model 263A potentiostat/galvanostat (Perkin Elmer, Bad Wildbad, Germany) in combination with the M270 software package. Cyclic voltammograms were recorded at scan rates of 20 mV s^{-1} over the potential range of 0 to 1000 mV. Experiments were carried out in a 5-ml cell placed on a magnetic stirrer. The supporting electrolyte was 0.05 M phosphate buffer, pH 7.0, 0.1 M $LiClO_4$, or 0.1 M borate buffer, pH 9.

3. Results and discussion

3.1. Biosensor architectures for rapid screening of mediator properties

In order to avoid significant loss of valuable redox mediators and enzymes, on one hand, biosensor architectures have to be designed in a way which allows a fast preparation procedure using different enzymes and redox mediators. On the other hand, the very different specific properties of enzymes applied in amperometric biosensors do not allow the choice of a single electrode architecture. This would possibly prevent productive interaction between a new redox mediator and a specific enzyme due to the inherent differences in the hydrophilicity and hydrophobicity of the enzymes, their stability in organic solution, the mass transport of the enzyme's substrate, etc.

In the present study, we have exclusively used O_2-independent redox enzymes which facilitates the interpretation of the obtained results. Two different classes of proteins were chosen. As representatives of the class of NAD^+-dependent dehydrogenases, we have used NAD^+-dependent

glucose dehydrogenase (NAD-GDH), NAD^+-dependent alcohol dehydrogenase (NAD-ADH), and NAD^+-dependent glycerol dehydrogenase (NAD-GlyDH). Since the coenzyme NAD^+ is not bound within the active site of these enzymes the coenzyme was added to the electrolyte solution prior to the measurements. The second class of enzymes contains as a primary redox site pyrroloquinolinequinone (PQQ) such as PQQ-dependent glucose dehydrogenase (PQQ-GDH) and quinohemoprotein alcohol dehydrogenase (QH-ADH). The latter is a multi-cofactor enzyme which possesses, in addition to the PQQ unit, at least four heme groups which are internally electrically connected to the primary redox site.

We have evaluated three different biosensor designs, all based on carbon paste with integrated redox mediators. Type A is based on the co-entrapment of the redox mediator and the enzyme within the carbon paste. Type B is prepared by adsorption of the enzyme on the surface of the mediator-modified carbon paste followed by the formation of a polyanionic Nafion membrane on top of the enzyme layer. Type C is prepared by entrapment of the enzyme within an electrochemically generated polymer hydrogel film on the surface of the mediator-modified carbon paste.

Carbon-paste based biosensors are well known, but the influence of factors such as the mode of immobilizing the enzymes on the stability of the biosensors obtained and the interaction with carbon-paste integrated redox mediators remains largely unexplored. Different environments are preferred by different enzymes for optimal performance; some enzymes tend to prefer a hydrophobic environment while some others may prefer an ionic environment.

These different sensor designs provide a hydrophobic environment for the enzyme in type A which may be advantageous for membrane enzymes, a polyanionic environment in type B which is supposed to especially support NAD^+-dependent enzymes, and a hydrogel entrapment which should be suitable for labile or hydrophilic enzymes. In addition, the immobilization of the enzyme in close proximity of the carbon-paste surface should, in principle, allow for a facile mass transport of the free-diffusing redox mediator into the enzyme layer, thus enabling fast electron transfer, at least in those cases where productive interaction between the enzyme and the redox mediator takes place.

In order to demonstrate the feasibility of the different architectures of biosensor design in facilitating electron transfer between different redox mediators and a variety of enzymes, we have used a family of Ru-complexes with variations in their ligand spheres. These Ru-complexes were of the type $[Ru(LL)_2X_2]$, where LL is either 1,2-bipyridine or 1,10-phenantroline and X is an acido ligand (see Scheme 1). Similar Ru-complexes have been previously reported as redox mediators with some oxidoreductases [13].

3.2. Electrochemical properties of ruthenium complexes

The different $[Ru(LL)_2X_2]$ mediators were integrated into a carbon paste and the redox properties were determined by means of cyclic voltammetry in $LiClO_4$ as electrolyte. Close to reversible redox behavior was encountered for all the different redox species, and as could be expected from the ligand properties, the redox potentials are rather high with the exception of compound **3** which has methyl-substituents at the bipyridyl residues. The redox potentials of the different mediators are presented in Table 1.

The incorporation of the redox mediators into the carbon-paste matrix did not affect their electrochemical properties, since the cyclic voltammograms of the mediator-enriched electrodes had similar characteristics as the cyclic voltammograms of the Ru-complexes recorded in solution.

The electron-transfer properties of the Ru-complexes **1**–**5** with some redox enzymes with integrated prosthetic groups and NAD^+-dependent enzymes were investigated. Consequently, the catalytic activity of the Ru-complex modified carbon-paste electrodes for the oxidation of NADH was evaluated by means of cyclic voltammetry. After addition of NADH in all cases, a significant catalytic oxidation wave was observed at the redox potential of the Ru-complex, proving unequivocally the ability of these compounds towards the electrocatalytic oxidation of NADH. An example is shown in Fig. 1 for compound **1**.

The electrocatalytic activity was estimated from the ratio of the anodic peak current of the Ru-complex modified carbon-paste electrode in the absence of NADH and the current after addition of 5 mM NADH. This ratio was found to be 29.42 ± 0.05 for compound **1**, 9.25 ± 0.05 for **2**, 20.82 ± 0.05 for **3**, 241.90 ± 0.05 for **4**, and 248.03 ± 0.05 for **5**. These figures show that the Ru complexes containing 1,10-phenantroline type ligands (**4** and **5**) are more efficient redox mediators for the oxidation of NADH. Although the complexes containing 2,2′-bipyridine type ligands could also mediate the oxidation of NADH, the current responses were lower for the same concentrations of NADH as compared to the current obtained at the 1,10-phenanthroline-complex-enriched electrodes.

3.3. Properties of the carbon-paste biosensors

Biosensors according to the proposed types A, B, and C were prepared for the evaluation of combinations of five different enzymes with the five Ru-complex mediators shown in Scheme 1. The properties of the biosensors were studied using constant potential amperometry at a working potential of 800 mV (vs. Ag/AgCl). This working potential

Table 1
Electrochemical properties of the Ru-complex mediators studied

	LL	X	R	*E* (mV vs. Ag/AgCl)
1	Bpy	Cl^-	H	522 ± 5
2	Bpy	SCN^-	H	532 ± 5
3	Bpy	Cl^-	Me	364 ± 5
4	Phen	CO_3^{2-}	H	472 ± 5
5	Phen	Cl^-	H	541 ± 5

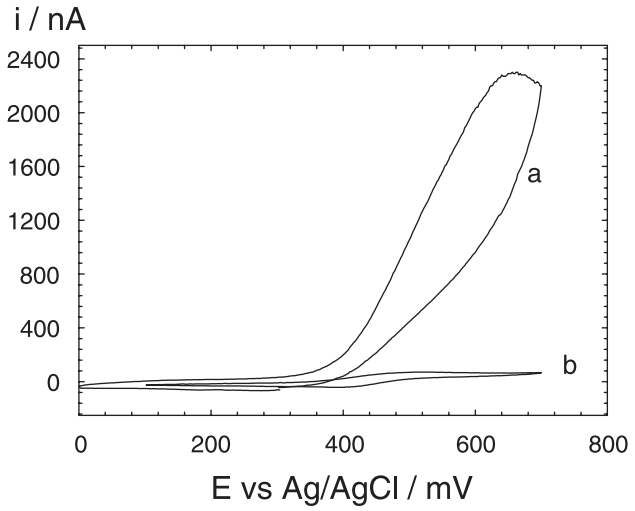


Fig. 1. Cyclic voltammograms recorded with a carbon-paste electrode enriched with Ru-complex **1** (a) in the presence of 5 mM NADH and (b) in the absence of NADH in 0.01 M $LiClO_4$. Scan rate = 20 mV s^{-1}.

is comparably higher than the redox potentials of all the mediators employed as shown in Table 1, to assure a diffusion limited oxidation of the redox mediator at the electrode in all cases. A constant background current was attained before aliquots of the enzyme's substrate were added stepwise. For biosensors based on NAD^+-dependent enzymes, 5 mM NAD^+ was added to the electrolyte solution prior to the measurements. Calibration curves were constructed from constant-potential amperometry experiments, and a typical example for an A-type biosensor containing redox mediator **5** within the paste, and modified with PQQ-dependent GDH, is shown in Fig. 2.

From the calibration curves for all combinations of enzyme, Ru-complex, and electrode type, the apparent Michaelis constant K_M^{app} and the maximal current were derived assuming pseudo Michaelis–Menten kinetics (Eq. (1)).

$$i = \frac{i_{max} \times S}{K_M^{app} + S} \quad (1)$$

where: i is the current, S the substrate concentration, K_M^{app} is the apparent Michaelis constant, and i_{max} is the maximum current at saturation concentration of the substrate. Table 2 shows the values of i_{max} and K_M^{app} obtained for each biosensor investigated in this study.

Obviously, the different redox complexes show a broad variation in their mediating properties for the different enzymes investigated. Moreover, the enzymes behaved differently depending on the immobilization procedure used. Compound **4**, for example, shows in general low electron-transfer activities with most enzymes which may be caused by the high solubility of this complex in aqueous solution. The best activity was encountered for compound **5** in combination with all enzymes investigated. As expected, the diffusion characteristics of the substrate to the active site of the enzyme are modulated by the immobilization type, which defines, on one hand, the hydrophilicity of the enzyme's environment and, on the other hand, the diffusion and partition properties within the sensing layer. For example, electrodes of type B exhibit an extended linear range due to the slower mass transport of the substrate through the overlaying Nafion film.

There are significant differences concerning the maximum current at substrate saturation for the different electrodes. A combination of mediator complex **4** and the type B electrode design gave rise to the best current response for NAD-ADH while for NAD-GlyDH, mediator complex **1** and the type B electrode design gave the best current. NAD-DGH gave rise to about the same current response with a combination of mediator complex **5** and electrodes of type A and type B. In general, PQQ-GDH and QH-ADH showed significantly higher currents as well as good conformity with the Michaelis–Menten kinetics equation irrespective of the mediator complex used when B-type electrode design was employed for PQQ-DGH and C-type electrode design for QH-ADH. Electrode designs of types A and B did not show distinct saturation points with QH-ADH, which prevented the determination of i_{max} and K_M^{app}, but saturation conditions were attainable using C-type electrodes. The significantly higher currents obtained for PQQ-DGH and QH-ADH can be explained by the fact that these proteins are membrane enzymes that are well accommodated within the hydrophobic environment established by the carbon-paste. Moreover, the differences in the turn-over rate of the enzymes and the comparatively slow redox conversion of enzymatically formed NADH may also play a part in the improved response of these enzymes over the related NAD^+-dependent enzymes.

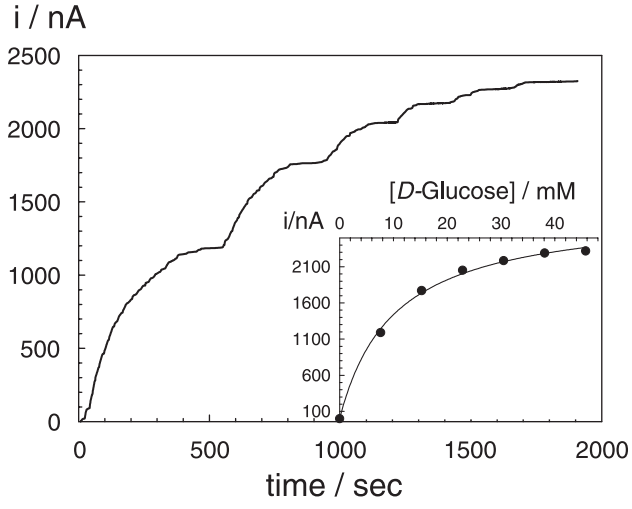


Fig. 2. Chronoamperometric response obtained for an A-type biosensor based on a carbon-paste electrode enriched with **5** (10 mg complex per 100 mg of carbon paste). The immobilized enzyme is PQQ-DGH, pH = 7.0, 50 mM phosphate buffer as supporting electrolyte, working potential 800 mV. Inset: Calibration curve for the same electrode.

3.4. Long-term stability

One of the most desirable features of an amperometric biosensor is its long-term operation and storage stability. The use of a biosensor is usually limited by the lifetime of the immobilized enzyme, and some of the electrodes were investigated under continuous amperometric measurements at 800 mV at room temperature for 7 days. As an example, the results obtained from carbon paste electrodes containing complex **5** and modified with quinoprotein-based enzymes using the B-type mode of electrode modification are shown in Fig. 3. Quinoproteins are well known to exhibit an improved "high" temperature resistance [18].

Table 2
Characteristics of the obtained sensors

Immobilised enzyme	Type of biosensor	Mediator	K^{app} (mM)[a]	i_{max} (nA)
NAD-ADH	A	**1**	59 ± 1	127 ± 20
NAD-ADH	B	**1**	320 ± 50	115 ± 9
NAD-ADH	C	**1**	280 ± 20	810 ± 30
NAD-GlyDH	A	**1**	49 ± 1	1110 ± 20
NAD-GlyDH	B	**1**	42 ± 2	1340 ± 20
PQQ–GDH	A	**1**	not determined	not determined
PQQ–GDH	B	**1**	51 ± 4	590 ± 10
PQQ–GDH	C	**1**	59 ± 9	235 ± 10
QH–ADH	B	**1**	not determined	not determined
QH–ADH	C	**1**	58 ± 2	6930 ± 7
NAD-GlycDH	A	**2**	23 ± 2	86 ± 2
PQQ–GDH	A	**2**	not determined	not determined
PQQ–GDH	B	**2**	5.3 ± 0.2	564 ± 2
QH–ADH	A	**2**	not determined	not determined
QH-ADH	C	**2**	320 ± 10	2446 ± 10
QH–ADH	B	**2**	not determined	not determined
NAD-ADH	A	**3**	37 ± 4	34.6 ± 0.1
NAD-GDH	A	**3**	160 ± 30	540 ± 7
NAD-GlyDH	A	**3**	not determined	not determined
PQQ–GDH	A	**3**	105 ± 9	290 ± 10
PQQ–GDH	B	**3**	536 ± 10	832 ± 10
QH–ADH	B	**3**	not determined	not determined
QH–ADH	C	**3**	64.3 ± 7	3750 ± 20
NAD-ADH	A	**4**	207 ± 20	430 ± 30
NAD-ADH	B	**4**	88 ± 3	797 ± 20
NAD-ADH	C	**4**	900 ± 20	139 ± 20
NAD-GDH	A	**4**	120 ± 20	1480 ± 10
NAD-GlyDH	A	**4**	1.25 ± 0.2	125 ± 8
PQQ–GDH	A	**4**	not determined	not determined
PQQ–GDH	B	**4**	1000 ± 20	590 ± 8
PQQ–GDH	C	**4**	35 ± 7	1310 ± 8
QH–ADH	C	**4**	360 ± 10	477 ± 10
NAD-ADH	A	**5**	49 ± 7	31 ± 1
NAD-ADH	B	**5**	47 ± 1	671 ± 10
NAD-ADH	C	**5**	250 ± 2	189 ± 1
NAD-GDH	A	**5**	63 ± 2	2270 ± 5
NAD-GDH	B	**5**	150 ± 5	730 ± 20
NAD-GlyDH	A	**5**	230 ± 20	99 ± 6
NAD-GlyDH	B	**5**	15 ± 1	26 ± 5
PQQ–GDH	A	**5**	25 ± 2	580 ± 10
PQQ–GDH	B	**5**	60 ± 3	93 ± 1
PQQ–GDH	C	**5**	117 ± 3	82 ± 1
QH–ADH	A	**5**	not determined	not determined
QH–ADH	B	**5**	not determined	not determined
QH–ADH	C	**5**	140 ± 7	2180 ± 40

[a] The standard deviation was calculated from three times the noise of the background current. 'not determined' represents calibration graphs which did not show a distinct saturation characteristic, thus not allowing to apply the Michaelis–Menten kinetics.

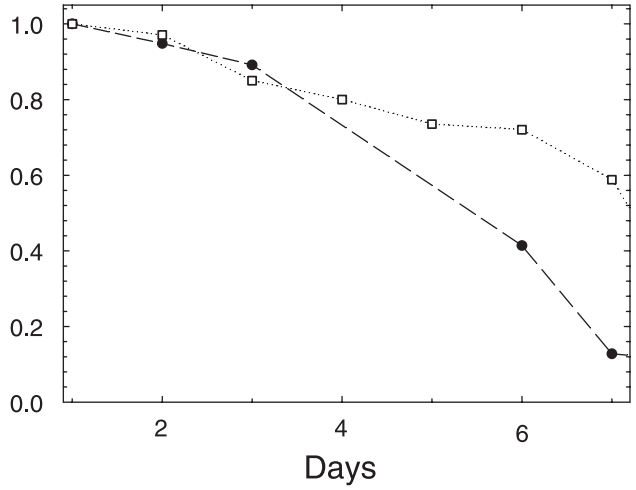


Fig. 3. Operation stability test of (□) A- and (●) B-type biosensors based on a carbon-paste electrode enriched with **5**. (Enzyme: PQQ-GDH; pH = 7.0; 50 mM phosphate buffer as supporting electrolyte at a working potential 800 mV).

As a stability parameter, the ability of the biosensor to reproduce the amperometric response over time was considered. As can be seen in Fig. 3, the A-type biosensor could still reproduce 60% of the initial current on the 7th day while only about 10% of the current could be retained in the case of the B-type biosensors. The possibility of a partial leakage of the enzymes in the case of the A-type biosensors into the buffer solution, resulting in a slow diffusion of "fresh" enzymes to the electrode surface, may be responsible for the higher stability of "A" type biosensors.

4. Conclusion

Ruthenium complexes of the general formula $Ru(LL)_2X_2$ were incorporated into carbon paste electrodes for the development of biosensors whose enzymes were immobilized using three different approaches designed to provide the enzymes with different environments. The performance of the biosensors when used for the oxidation of some oxidoreductases such as NADH was evaluated in conjunction with the mode of immobilizing the enzymes onto the electrodes. The electrochemical properties of the biosensors varied significantly, depending on the method used to immobilize the enzymes as well as the ruthenium complex present in the bulk of the base carbon paste electrode. This indicates that the method used to immobilize the enzymes unto the biosensors plays a crucial role in determining the nature of the environment of the enzyme, hence its performance and stability. In addition, the proposed carbon-paste

based redox-mediator test system provides a suitable tool for a first evaluation of different redox mediators together with a variety of different enzymes, taking into account the different nature of the sensor compounds.

Acknowledgements

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References

[1] A.L. Ghindilis, P. Atanasov, E. Wilkens, Enzyme-catalyzed direct electron transfer: fundamentals and analytical applications, Electroanalysis 9 (1997) 661–675.
[2] K. Habermüller, M. Mosbach, W. Schuhmann, Electron-transfer mechanisms in amperometric biosensors, Fresenius' J. Anal. Chem. 366 (2000) 560–568.
[3] A.E.G. Cass, G. Davis, G. Francis, O. Hill, W. Aston, I. Plotkin, L. Scott, A.A. Turner, Ferrocene-mediated enzyme electrode for amperometric determonation of glucose, Anal. Chem. 56 (1984) 667–671.
[4] V.R.A. Marcus, Elektronentransferreaktionen in der Chemie, Theorie und Experiment (Nobel-Vortrag), Angew. Chem. 8 (1993) 1161–1280.
[5] C. Bourdillon, C. Demaille, J. Moiroux, J.-M. Savéant, New insights into the enzymatic catalysis of the oxidation of glucose by native and recombinant glucose oxidase mediated by electrochemically generated one-electron redox cosubstrates, J. Am. Chem. Soc. 115 (1993) 2–10.
[6] K. Habermüller, W. Schuhmann, A low-volume electrochemical cell for the deposition of conducting polymers and entrapment of enzymes, Electroanalysis 10 (1998) 1281–1284.
[7] K. Kalcher, J. Kauffmann, J. Wang, I. Svancara, K. Vytras, C. Neuhold, Z. Yang, Sensors based on carbon-paste in electrochemical analysis—a review with particular emphasis on the period 1990–1993, Electroanalysis 7 (1995) 5–22.
[8] L. Gorton, Carbon-paste electrodes modified with enzymes, tissues, and cells, Electroanalysis 7 (1995) 23–45.
[9] S. Zhang, G. Wright, Y. Yang, Materials and techniques for electrochemical biosensor design and construction, Biosens. Bioelectron. 15 (2000) 273–282.
[10] I. Svancara, K. Vytras, J. Barek, J. Zima, Carbon paste electrodes in modern electroanalysis, Crit. Rev. Anal. Chem. 31 (2001) 311–345.
[11] A.B.P. Lever, Electrochemical parametrization of metal complex redox potentials using the Ru(III)/Ru(II) couple to generate a ligand electrochemical series, Inorg. Chem. 29 (1990) 1271–1285.
[12] A.D. Ryabov, Yu.N. Firsova, M.I. Nelen', Ferrocenium salts as true substrates of glucose oxidase, Appl. Biochem. Biotechnol. 61 (1996) 25–37.
[13] A.D. Ryabov, Yu.N. Firsova, V.N. Goral, V.S. Sukharev, A.Yu. Ershov, C. Lejbolle, M. Bjerrum, A.V. Eliseev, Horseradish peroxidase-catalysed oxidation of *cis*-[Ru(LL)$_2$XY] complexes by hydrogen peroxide (LL=2,2′-bipyridine and 1,10-phenanthroline): equilibria, kinetics, mechanism, and active site reassembly, Inorg. React. Mech. (2001) 1–18.
[14] E.S. Ryabova, V.N. Goral, E. Csöregi, B. Mattiasson, A.D. Ryabov, Coordinative approach to mediated electron transfer: ruthenium complexes to native glucose oxidase, Angew. Chem., Int. Ed. Engl. 38 (1999) 804–807.
[15] S. Reiter, K. Eckhard, A. Blöchl, W. Schuhmann, Redox modification of proteins using sequential-parallel electrochemistry in microtiter plates, Analyst 126 (2001) 1912–1918.
[16] A.D. Ryabov, Yu.N. Firsova, A.Yu. Ershov, I.A. Dementiev, Spectrophotometric kinetic study and analytical implications of the glucose oxidase-catalysed reduction of $[M(III)(LL)_2Cl_2]^+$ complexes by D-glucose (M=Os and Ru, LL=2.2′-bipyridine and 1,10-phenantroline type ligands), J. Bioinorg. Chem. 4 (1999) 175–182.
[17] A.D. Ryabov, V.S. Sukharev, L. Alexandrova, R. Le Lagadec, M. Pfeffer, New synthesis and new bio-application of cyclometalated ruthenium(II) complexes for fast mediated electron transfer with peroxidase and glucose oxidase, Inorg. Chem. 40 (2001) 6529–6532.
[18] L. Marcinkeviene, I. Bachmatova, R. Seemanaite, R. Rudomanskis, G. Braenas, R. MešKene, R. MešKus, Mediated and reagentless glucose biosensors based on PQQ-dependent glucose dehydrogenase from *Erwinia sp.* 34-1, Biologija 1 (1998) 15–17.
[19] L. Marcinkeviciene, I. Bachamatova, R. Semenaite, R. Rudomanskis, G. Brazenas, R. Meskiene, R. Meskus, Purification and characterisation of *PQQ*-dependent glucose dehydrogenase from *Erwinia* sp. 34-1, Biotechnol. Lett. 21 (1999) 187–192.
[20] N.I. Veletsky, I.A. Dementiev, A.Y. Ershov, A.B. Nikol'skii, Photochemistry of bridged symmetrical polypyridyl ruthenium(II) complexes, J. Photochem. Photobiol., A Chem. 89 (1995) 99–103.
[21] T/US00/16247 BASF; EP 0256521 B1 BASF.
[22] C. Kurzawa, A. Hengstenberg, W. Schuhmann, Immobilization method for the preparation of biosensors based on pH shift induced deposition of biomolecule-containing polymer films, Anal. Chem. 74 (2002) 355–361.
[23] B. Ngounou, S. Janiak, C. Kurzawa, J. Wylamrzy, A. Golloch, W. Schuhmann, Elektrochemisch induzierte pH-Wert Modulation und lokalisierte Abscheidung von Polymeren als Basis für die Entwicklung amperometrischer Biosensoren, in: J.P. Baselt, G. Gerlach (Eds.), 5. DSS, Dechema-Monographien"Dresdner Beiträge zur Sensorik", Band 16, Sensoren im Fokus neuer Anwendungen, 2002, pp. 121–128. w.e.b., Dresden.