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Reagentless Hydrogen Peroxide and L-Lactate Sensors Based on Carbon Paste Electrodes Modified with Different Peroxidases and Lactate Oxidases

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Abstract. Reagentless amperometric hydrogen peroxide and L-lactate electrodes based on bienzyme modified carbon pastes were tested and compared. The behaviour of 7 peroxidases and two lactate oxidases immobilized in carbon pastes were investigated amperometrically under flow injection conditions. By the combination of recombinant lactate oxidase from *Aerococcus viridans* (RLOD) and lactate oxidase from *Pedicoccus sp.* with (LOD) horseradish peroxidase, peroxidase from *Arthromyces ramosus*, microperoxidases MP-8 and MP-11 sensitive carbon paste electrodes were prepared to detect H_2O_2 and L-lactate. The selectivity with respect to several redox active substances and substrates of the peroxidases is slightly influenced by the selection of the heme containing peroxidase. The operational stability of both the hydrogen peroxide and the L-lactate sensors could be improved by electrochemical deposition of *o*-phenylenediamine onto bienzyme modified carbon paste electrodes.
Despite microperoxidases have a considerably lower electrocatalytical efficiency per molecule, especially MP-8 can be used to modify carbon paste electrodes for sensitive H_2O_2 detection. Both MP-8 and MP-11 can be coimmobilized with LOD and RLOD to detect L-lactate.

In a number of recent reports bienzyme modified carbon pastes have been prepared by coimmobilization of hydrogen peroxide producing oxidases with horseradish peroxidase (HRP) [1–6] to produce mediatorless amperometric biosensors. These biosensors detect H_2O_2 and the substrate of the oxidase at low potentials between –200 and +50 mV *vs.* Ag/AgCl largely excluding the influence on the response from easily oxidizable interferences. Recently Johansson *et al.* [3] compared the use of peroxidase from *Arthromyces ramosus* (ARP) and Horseradish peroxidase (HRP) coimmobilized with D-amino acid oxidase in carbon paste electrodes (CPEs). There are other peroxidases with very different pH optima [7] opening a convenient way to adapt the peroxidase catalysed H_2O_2 reduction to the pH optima of the coimmobilized oxidases.

Ruzgas *et al.* [8], Kulys *et al.* [9], Spohn *et al.* [10] and Lindgren *et al.* [11] have shown for the oxidized forms of different peroxidases that substrates, see *e.g.* phenol, its derivatives, and aromatic amines can interfere with the response acting as electron donor producing electrochemical active species acting as redox mediators, thus competing with the electrode as the source of electrons. However, peroxidases from different sources have different selectivity patterns in regard to their electron donors opening a way to tune the selectivity of peroxidase/oxidase modified CPEs, *e.g.* of the earlier described L-lactate electrodes [10, 12, 13].

Protoporphyrine IX containing proteins, *e.g.* HRP [1, 14, 15], ARP [3, 16], lactoperoxidase (LRP) [16, 17], chloroperoxidase (CRP) [18], catalase (CAT) [17], cytochrome c peroxidase [19, 20] and heme containing polypeptides, *e.g.* microperoxidases, MP-9 [21] and MP-11 [22, 23] catalyse the electrochemical reduction of H_2O_2 at different electrode materials, *e.g.* gold [24], tin dioxide coated glass [21], glassy carbon [16] and graphite [16, 25] in a mediatorless fashion. Up to now carbon paste electrodes have been reported modified with HRP [3, 5], ARP [3] and MP-11 [22, 23] and used for mediatorless sensing of H_2O_2.

Adams and Gould [26] discussed a reaction sequence, which seems to explain this electrocatalytic phenomenon. According to this mechanism, in a first step H_2O_2 oxidizes native peroxidase in a single two electron reaction with the formation of an iron-oxo-porphyrin cation radical denoted compound I ($[(HN)\ Fe^{IV}{=}O]^{\cdot+}$), which can be observed for all ferriprotoporphyrin IX containing peroxidases. The rereduction of compound I takes place in two discrete one electron steps at the electrode. In the first step compound I accepts one electron and is transferred into compound II ($[(HN)Fe^{IV}{=}O]^{\cdot+}$), which in turn accepts the necessary second electron. The aim of this work was to investigate the effect of coimmobilizing different lactate oxidases with peroxidases other than HRP, such as microperoxidases 8 and

11(MP-8, MP-11), catalase, and chloroperoxidase in carbon pastes to prepare new bienzyme modified paste electrodes.

Experimental

Chemicals and Reagent

HRP (EC 1.11.1.7, type VI, P8375, 280 U/mg), MP-8 (M4757), MP-11 (M6756), lactoperoxidase (LRP, EC 1.11.1.7, L2005, 103 U/mg), chloroperoxidase from *caldariomyces fumago* (CRP, EC 1.11.1.10, C0887, 1200 U/mg), recombinant NADH peroxidase from *Streptococcus faecalis* (NRP, EC 1.11.1.1, N0895, 170 U/mg), LOD from Pediococcus sp. (L0638, 40 U/mg), polyethylenimine (PEI, 50% m/m aqueous solution, molecular weight 50 000 Da, P3143), *o*-phenylendiamine (*o*-PDA, P9029) and bovine serum albumin (BSA, A7638) were from SIGMA (main catalog 1994, St.Louis, MO, USA). Peroxidase from *Arthromyces ramosus* (ARP, EC 1.11.1.7, lot no. 900511, 250 U/mg) was obtained from Suntory Ltd., Japan. The above mentioned chemicals were used as received. Catalase from beef liver (CAT, EC 1.11.1.6, 65 000 U/mg, Boehringer Mannheim, Mannheim, Germany) was purified by diaultrafiltration across a membran microcell (30 000 Da, Amicon, Witten, Germany). Two portions of 400 µl of the enzyme suspension were processed. After each of the first three filtration steps the enzyme was resuspended in 0.1M potassium/sodium phosphate buffer ($KNaP_i$), pH 7.0. In the last filtration step the volume of the enzyme solution was reduced to 100 µl. Lactate oxidase from *Aerococcus viridans* (RLOD, 1380 U/mg) was provided by Genzyme Diagnostics (West Malling, Kent, England).

Silicon oil (C719, 50% phenylmethylsilicon) was from Alltech (Arlington Heights, IL, USA). Graphite powder (cat. no. 50 780) was from Fluka (Buchs, Switzerland). All other chemicals (Merck, Darmstadt, Germany) were of analytical grade and used without further purification. 0.1M citrate/$KNaP_i$ buffers between pH 4.0 and 6.0, 0.1M $KNaP_i$ buffers between pH 6.0 and 8.0, and 0.1M Tris/H_2SO_4 buffers between pH 8.0 and 9.5 were used to adjust different pH values of the carrier stream in the flow detector cell.

Preparation of the Carbon Paste Electrodes

The enzyme-modified carbon pastes have been prepared as described earlier [2, 7] by adsorption of the enzymes onto graphite powder (GP), which was first heat treated at 700 °C for 15 s and cooled back to room temperature in a desiccator in the presence of blue gel before further use.

100 mg of graphite powder were suspended in 400 µl of enzyme solution in 0.1M KP_i buffer. To produce bienzyme-modified CPEs this solution was prepared by mixing 100 µl of enzyme solution containing 1 mg of the corresponding lactate oxidase and 1 mg of one of the peroxidases in 0.1M KP_i buffer, pH 8.0 with 300 µl of an aqueous 0.2 m/m% aqueous polyethylenimine solution, which was adjusted with concentrated phosphoric acid to pH 8.0. To prepare peroxidase modified CPEs 1 mg of a peroxidase was mixed with the same buffer/polyelectrolyte solution.

The mixture was allowed to react at 4 °C for 3 h in all cases before drying under water jet vacuum for 4.5 h. The carbon pastes were prepared by mixing the resulting powder with 40 µl silicon oil and filled into plastic syringe holders (1 ml syringe, ONCE, Rodby, Denmark) leaving 3–4 mm empty in the tip to be filled with the enzyme modified CP. The electrode tip was gently rubbed on fine paper to produce a flat surface. Some of the CPEs were modified by electrochemical polymerization of *o*-phenylendiamine (*o*-PDA) under flow injection conditions according to Palmisano *et al.* [27] at a deposition potential of + 650 mV *vs.* Ag/AgCl. 50 µl of 5 mM *o*-PDA dissolved in 0.2 mM sodium acetate buffer, pH 5.2 were injected 9 times into the carrier buffer, 0.1M $KNaP_i$, pH 7.0 of the FIA set-up flowing with 0.6 ml min^{-1}. All enzyme modified carbon paste electrodes (EMCPE) were stored at 4 °C.

Measuring Set-up

The carbon paste electrodes (CPE) were mounted in a flow through cell of the confined wall-jet type [28]. An Ag/AgCl electrode in 0.1M KCl and a Pt wire were used as the reference and the auxiliary electrode, respectively. The electrodes were connected to a potentiostat (Zäta Electronics, Lund, Sweden).

The wall-jet cell was inserted into a FIA set up consisting of a peristaltic pump (Gilson Minipuls 2, Villier-le-Bel, France) and a pneumatically operated injection valve (Cheminert, type SVA, valco Instruments, Houston, TX, USA) with an injection loop of 50 µl. The injection valve and the wall-jet cell were connected by a knotted PTFE tubing with a length of 30 cm and an inner diameter of 0.5 mm.

The carrier solution was propelled with a flow rate of 0.6 ml min^{-1}. Each measuring result is the mean average of three to four injection peaks implemented for each electrode among the three prepared from the same carbon paste batch. The dispersion factor D_{max} was determined as described earlier according to Ruzicka and Hansen [29] to be 1.3.

Results and Discussion

Carbon Paste Electrodes Modified with Various Peroxidases

The early reports on mediatorless electron transfer between carbon electrodes and peroxidases revealed that a reduction current was noticed in the presence of H_2O_2 close to the formal potentials ($E^{o'}$) of compound **I**/compound **II**/native HRP, that steadily increased as the potential was made more negative, and started to level off at around – 100 mV *vs.* Ag/AgCl [14, 16, 30–32]. The $E^{o'}$ of compound **I**/compound **II** and compound **II**/native HRP are very close at neutral pH and found slightly more positive than + 600 mV *vs.* Ag/AgCl. From these observations it became clear that peroxidase modified electrodes could be used for sensing H_2O_2 within a potential range much more negative compared with direct electrochemical oxidation of H_2O_2 at conventional unmodified electrodes (usually needing at least + 600 mV) with anticipated much less influence from easily oxidizable interfering compounds present in "real" samp-

les. The background current and noise levels are expected to be lower at lower applied working potentials.

It should be noted that some oxidases, especially the oxygen-specific enzymes [33] choline oxidase and cholesterol oxidase and alcohol oxidase from different sources [34] react only slowly with artificial mediators.

Hydrodynamic voltammograms were therefore recorded under flow injection conditions in the presence and in the absence of 0.5 mM H_2O_2 to investigate the variation of the background current and the mediatorless response to H_2O_2 with the applied potential. Fig. 1 shows the voltammograms for CP electrodes modified with the various peroxidases or with BSA. As in previously published investigations the HRP and ARP modified CPEs show a high electrocatalytic H_2O_2 reduction current starting around 420 and 580 mV, respectively. A control experiment with a BSA modified CPE showed that it is specifically the peroxidase and not an effect of just protein that causes the electrocatalytic effect. As expected the BSA modified CPE did not show any electrocatalytic response to H_2O_2 and only a small anodic current was registered at applied potentials of around + 400 mV. The CPEs modified with LRP, CRP, and NRP did not produce any significant electrocatalytic response to H_2O_2 despite previously reported electrocatalytical activity for solid graphite electrodes modified with LRP [16] and CRP [18]. Catalase however, caused a significant reduction current to appear at a potential of around + 450 mV indicating electrocatalytical activity at the interface between the CPE and the sample solution. Tatsuma *et al.* [17] demonstrated recently a similar electrocatalytic activity of catalase immobilized in a polythiophene film coated on tin dioxide electrode and explained this with a direct electron transfer between electrode and enzyme. Wang *et al.* [35] reported a similar effect of immobilized catalase in organic solvents. For the microperoxidase modified CPEs as shown in Fig. 1 H_2O_2 reduction starts at + 330 mV for MP-8 and at + 250 mV for MP-11 modified CPE. This is in accordance with previous reports by Csöregi *et al.* [16], Razumas *et al.* [22, 23], and Lötzbeyer *et al.* [24] for solid graphite, gold, and carbon paste electrodes, respectively, modified with MP-11 revealing an efficient electrocatalytic reduction of H_2O_2 starting at potentials around + 400 mV *vs.* Ag/AgCl. A more positive potential would be expected for MP-8 modified CPEs since the protoporphyrine centre of this microperoxidase has a lower degree of shielding by the peptide chain than MP-11. Comparing the lower molecular weights of MP-8 (1526 Da) and MP-11 (1881 Da) with those of HRP (40 500 Da) and ARP (41 000 Da) an important lower catalytic efficiency with respect to the number of added heme centres can be concluded.

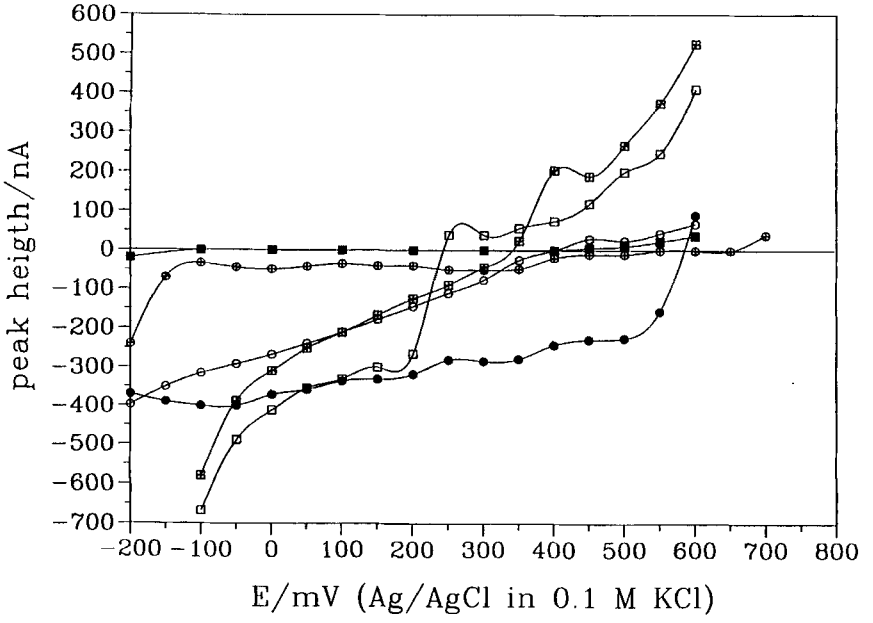

Fig. 1 Hydrodynamic voltammograms recorded at CPEs modified with different peroxidases under FI conditions for 0.5 mM H_2O_2, pH 7.0, 0.1 M KP_i, 25 °C, flow rate 0.58 ml min^{-1}, and injection volume 50 µl: – ■ – BSA, – ⊕ – CAT, –⊞ – MP-8, – □ – MP-11, – ● – ARP, – ○ – HRP.

In Fig. 2 the variation of the background current with the applied potential is shown. As can be seen between – 100 and + 20 mV the background current switches sign reflecting that within this potential range the background takes its lowest values and that the noise level for these electrodes should be minimal.

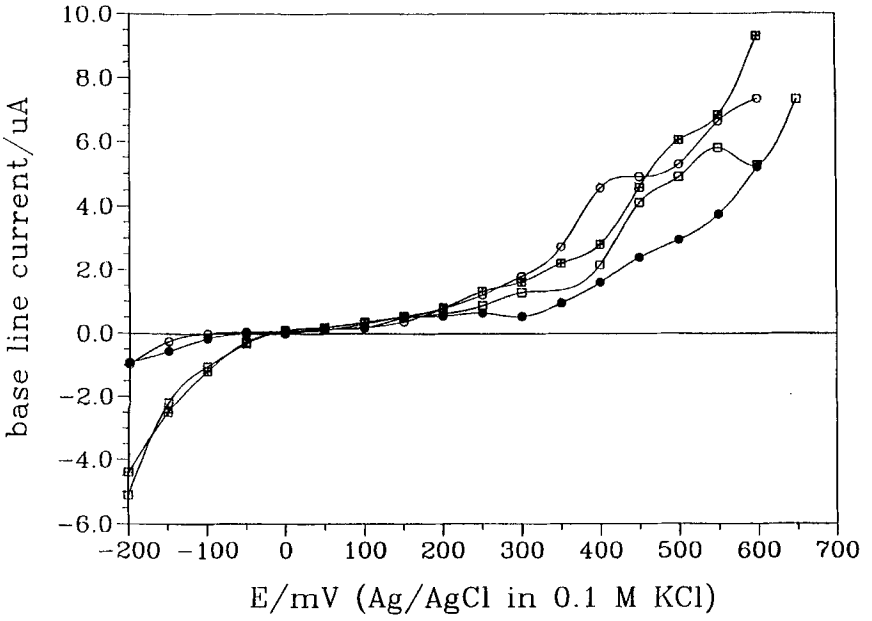

Fig. 2 Dependencies of the base line current of CPEs, modified with different peroxidases, on the electrode potential, measuring conditions and symbols as in Fig.1

Because the most efficient electrocatalytic reduction of H_2O_2 is achieved by modification of the electrodes with HRP, ARP, MP-8, and MP-11 modified electrodes, these enzymes were coimmobilized with the lactate oxidases to produce electrodes with an acceptable L-lactate sensitivity in the potential range between −50 and +200 mV *vs.* Ag/AgCl. The hydrodynamic voltammograms (not shown) recorded for 1 mM L-lactate were very similar to those recorded for the H_2O_2 detection.

Substrate Response as a Function of pH

As the response of the electrodes is based on three consecutive reactions [1, 2, 26] with anticipated different pH profiles the response to both lactate and H_2O_2 were registered at −50 mV as well as the background current of the bienzyme modified CPEs between pH 4 and 9. Tab. 1 summarizes pH values for which maximum lactate and H_2O_2 responses were obtained. For all bienzyme modified CPEs response currents were obtained *in the pH range* investigated. HRP modified CPEs showed a very broad pH profile for detection of H_2O_2 between pH 4.5 and 7.0 and the sensitivity slightly increased when decreasing the pH from 7.0. ARP modified CPEs had a clear pH optimum between pH 4.5 and 6.5. The MP-8 and MP-11 modified CPEs had pH profiles similar to those recorded for the HRP modified CPEs. However, the sensitivities more strongly increase with decreasing the pH. The microperoxidase modifed CPEs show very unstable detection signals at pH values lower than 7.0.

Tab. 1 pH optima of the bienzyme modified carbon paste electrodes with respect to the H_2O_2 – and the L-lactate sensitivity and the resulting ratios I_L/I_H of the lactate to H_2O_2 sensitivity, I_L - peak signal height in nA/mM L-lactate, I_H – the same for H_2O_2

	pH optimum		I_L/I_H
	H_2O_2 detection	lactate detection	
LOD/HRP	3.5 – 4.5	7.0 – 7.5	0.25
LOD/ARP	5.8 – 7.0	7.0 – 8.0	0.14
LOD/MP-8	4.5 – 5.5	6.0 – 7.0	0.10
LOD/MP-11	4.5 – 6.0	6.5 – 7.5	0.27

The lactate response showed a surprisingly high degree of independence of pH. The HRP and ARP-CPEs provided stable H_2O_2 and lactate signals between pH 6.0 and 9.0. As expected from the pH optimum of dissolved LOD found between pH 6.0 and 7.0 and its greatest pH stability between 7.0 and 9.0 [36], the maximum lactate sensitivity was achieved at slightly higher pH values between pH 7.0 and 8.0.

The highest ratios between the lactate and the H_2O_2 sensitivities (Tab. 1) are found for the HRP/LOD and the MP-11/LOD modified electrodes. The LOD/MP-8 and the RLOD/MP-8 modified CPEs showed slightly greater peak widths for the lactate detection compared with the other bienzyme modified CPEs. The coimmobilization of RLOD with the selected peroxidases had no significant influence on the pH dependence of the lactate response(data not shown).

Background Current as a Function of Paste Composition and pH

With respect to the signal to noise ratios the variation of the base line currents with pH is essential. The background current was found to increase strongly with decreasing pH for all EMCPEs. This can be explained by the redox potentials of O_2/H_2O and of the quinoid surface groups, which are shifted to more positive values with decreasing pH. This effect may be promoted by the increased protonation of the negatively charged surface groups, *e.g.* carboxyl groups and the increased pH gradient between the CPE surface and the carrier solution. The isoelectric point of the oxidized graphite surface is found around pH 5.6 according to Armstrong *et al.* [37]. A considerably weaker shielding of the electrode surface by the positively charged polyelectrolyte/protein complexes follows at a pH lower than 5.6. At a pH lower than 7.0 microperoxidases modified CPEs showed much higher background currents than HRP and ARP-CPEs. It should be noted that ARP modified CPEs showed excellent baseline behaviour throughout the pH range between 4.0 and 9.0 with the lowest background currents smaller than 5 nA. The background currents shifted by 40 or 60 nA to more negative values when replacing ARP for HRP. Microperoxidase modified CPEs showed nonacceptable baseline instabilities at pH < 6.0. The MP-11 modified CPEs had relatively low positive baseline currents around +30 nA in the alkaline range between pH 7.0 and 9.5. For MP-8 modified CPEs background currents between −80 and −100 nA were registered for pH values higher than 7.5. Therefore microperoxidases can be applied to prepare bienzyme modified CPEs giving stable H_2O_2 and L-lactate signals at pH > 7.0.

Operational Stability

Tab. 2 summarizes the signal stabilities for L-lactate of selected EMCPEs measured for 48 h under FIA conditions. The LOD/HRP and the LOD/ARP modified electrodes showed a considerably better signal stability than the microperoxidase modified CPEs. The replacement of LOD for RLOD caused a significant decrease in the signal stability. The stabilizing effect of PEI is significant for all enzyme combinations. Because of their fully dissociated and weakly shielded carboxylate groups the microperoxidases interact stronger with PEI in comparison with the neutral HRP having an isoelectric point between 7.0 and 8.0, which is the main component in the applied enzyme preparation [38]. In the absence of PEI very unstable CPEs were obtained with rapidly decreasing sensitivities. However, to explain the considerably higher operational stability of the HRP and ARP modified CPEs compared with the microperoxidase modified CPEs, additional stabilizing factors, *e.g.* hydrophobic interactions between the enzyme and the graphite particles and a multipoint chelating effect by PEI are to be taken into consideration. The smallest difference between the pH optima of the H_2O_2 and the L-lactate detection and the strong interaction of PEI with ARP, caused mainly by its very low isoelectric point $pH_{iso} = 3.4$ [39] may be the reason of the highest operational stability of the LOD/ARP modified CPE. The stability of all lactate sensors can be significantly improved by the electrochemical deposition of a *o*-PDA layer as shown in Tab. 2 and is expected from previous investigations [2, 6, 27]. The *o*-PDA layer prevents the dissolution of the enzymes from the paste and the direct contact of the enzymes with the interfacial layer between the organic solvent phase of the CPE and the aqueous carrier solution. These factors would otherwise cause a more rapid decrease of the immobilized enzyme activities. Sasso *et al.* [40] demonstrated, that a *o*-PDA layer decreases the influence of redoxactive substances, *e.g.* ascorbic acid, uric acid, and L-cysteine. This effect is confined by own measurements on *o*-PDA coated HRP/LOD modified CPEs. Johansson *et al.* [6] used *o*-PDA coated EMCPEs in direct contact with complex sample matrix of fermentation broths to determine ethanol demonstrating as like as Sasso *et al.* [40] the partial or complete elimination of the electrode fouling by proteins and possibly other polymeric substances.

Tab. 2 Operational stability of selected bienzyme modified carbon paste electrodes (BMCPE) under flow injection conditions with respect to the L-lactate detection, 4 injections of 40 µl of 1 mM L-lactate per hour, I – measured peak height, I_0 – peak height at the beginning of the stability test, all other measuring conditions as in Fig. 1.

BMCPE	relative L-lactate response I/I_0 after 48 h	
	without *o*-PDA layer	with *o*-PDA layer
RLOD/HRP	0.78	not measured
LOD/HRP	0.83	0.92
LOD/ARP	0.92	0.96
LOD/MP-11	0.25	0.43
LOD/MP-8	0.60	0.72

Response Ranges and Sensor Characteristics

Eight selected EMCPEs were compared based on the results described above. One series of electrodes was based on LOD coimmobilized with HRP, ARP, MP-8 or MP-11 and another series based on RLOD coimmobilized with the same peroxidases. As shown in tab. 3 the signal currents can be correlated with the injected concentrations by either linear equations or Hanes plots (apparent Michaelis–Menten-Kinetics) in the ranges

Tab. 3 Correlation of the signal current I and the injected concentration c by linear calibration graphs (lp) and electrochemical Hanes plots (Hp) for the enzyme modified carbon paste electrodes (EMCPE), repectively, confidence limit $\alpha = 0.05$, n = 4, m = 8, r correlation coefficient.

EMCPE	regression line	r	plot
	H_2O_2 - detection in the range 0.05 – 1.0 mM		
MP-8	$I = (343.7 \pm 6.2)\ nAmM^{-1} \cdot c + (12.4 \pm 3.7)\ nA$	0.999	lp
MP-11	$I = (84.4 \pm 4.5)\ nAmM^{-1} \cdot c + (4.8 \pm 2.5)\ nA$	0.999	lp
FRP	$c/I = (1.21 \pm 0.24)\ nA^{-1} \cdot c + (1.10 \pm 0.13)\ \mu M/nA$	0.990	Hp
HRP	$c/I = (2.25 \pm 0.25)\ nA^{-1} \cdot c + (1.04 \pm 0.15)\ \mu M/nA$	0.997	Hp
	lactat detection in the range 0.1 – 10 mM		
LOD/MP-8	$c/I = (1.05 \pm 0.16)\ nA^{-1} \cdot c + (5.23 \pm 0.77)\ \mu M/nA$	0.972	Hp
LOD/MP-11	$I = (16.4 \pm 1.9)\ nAmM^{-1} \cdot c + (17.7 \pm 8.5)\ nA$	0.990	lp
LOD/FRP	$c/I = (1.48 \pm 0.13)\ nA^{-1} \cdot c + (4.09 \pm 0.58)\ \mu M/nA$	0.988	Hp
LOD/HRP	$c/I = (1.46 \pm 0.14)\ nA^{-1} \cdot c + (2.47 \pm 0.51)\ \mu M/nA$	0.990	Hp
RLOD/MP-8	$I = (68.5 \pm 5.3)\ nAmM^{-1} \cdot c + (67.9 \pm 23.8)\ nA$	0.996	lp
RLOD/MP-11	$I = (25.1 \pm 1.0)\ nAmM^{-1} \cdot c + (18.1 \pm 4.7)\ nA$	0.999	lp
RLOD/FRP	$c/I = (2.04 \pm 0.59)nA^{-1} \cdot c + (15.13 \pm 2.65)\ \mu M/nA$	0.888	Hp
RLOD/HRP	$c/I = (4.06 \pm 0.17)\ nA^{-1} \cdot c + (8.44 \pm 0.78)\ \mu M/nA$	0.997	Hp

from 0.05 to 1 mM H_2O_2 and from 0.1 to 10 mM lactate. There is no significant difference between LOD and RLOD in their influence on the H_2O_2 sensitivity. With the exception of MP-11 modified CPEs all of the selec-ted EMCPEs show a high H_2O_2 sensitivity with detection limits lower than 1 µM. MP-8 and MP-11 modified CPEs show a higher degree of linearity for both H_2O_2 and lactate detection than the CPEs modified with the other peroxidases. This can be explained by the higher number of electrocatalytically active centres on the electrode surface. This is supported by the greater apparent electrochemical Michaelis–Menten constants K_M^{app} for both H_2O_2 and L-lactate of the microperoxidase modified CPEs. No essential differences between the lactate sensitivities were found for EMCPEs modified with LOD. The situation is similar for the corresponding RLOD modified CPEs. In both series of electrodes the MP-11 modified CPEs showed the lowest sensitivity. The results demonstrate that there is no significant influence of the selected lactate oxidase on the lactate sensitivity. Because the lactate sensitivity in both series of electrodes was not essentially influenced by the selected peroxidase with the exception of MP-11 modified CPEs, the lactate response is kinetically determined by the conversion of lactate in these cases. Because both the LOD/MP-11 and the RLOD/MP-11 modified CPEs show a significantly higher degree of linearity compared to the other CPEs the lactate detection for these electrodes seems to be dominated by the MP-11 catalyzed H_2O_2 detection. For both HRP modified CPEs linear Hanes-plots were obtained for both H_2O_2 and lactate (Tab. 3). The LOD/ARP modified CPE showed a linear Hanes plot for H_2O_2 detection but a nonlinear Hanes plot for lactate. The lactate detection of the RLOD/MP-8 modified CPE can be approximately described by a linear calibration graph.

It should be noted that the lactate responses of the HRP and ARP modified CPEs significantly depend on the amount of immobilized LOD taken to 100 mg of graphite powder and are found to increase up to a maximum amount of 4 mg of LOD [10]. For the fixed amount of 1 mg of LOD used in this work the lactate response was dependent on the amount of immobilized peroxidase and also found to increase up to 2 mg of peroxidase/100 mg of graphite powder. Therefore, the lactate response is primarily controlled by the LOD catalysis.

All HRP and ARP modified CPEs have response times, during which 90% of the steady state signal is reached, shorter than 15 s for concentration steps from 0 to 0.1 mM H_2O_2 and shorter than 15 s for steps from 0 to 1 mM lactate. The corresponding signal peaks do not show any significant peak tailing. The MP-8 and MP-11 modified CPEs show slightly longer response times and a significant peak tailing for the lactate detection.

Selectivity

To determine the selectivities of the EMCPEs the influence of redox active substances, peroxidase substrates, and possible LOD substrates were investigated.

Tab. 4 compares the influence of some α-hydroxy- and ketocarboxylic acids on the lactate response of the RLOD and LOD modifed CPEs. The RLOD/HRP-CPE and the LOD/HRP-CPE showed very similar selectivity patterns. The RLOD modified CPEs have a slightly better L-lactate selectivity against glycolate and L-phenyllactate than the LOD/HRP-CPEs.

Tab. 4 Selectivity of LOD/HRP and RLOD/HRP modified CPEs, normalized signals in %.

	in the absence of L-lactate		in the presence of 1 mM L-lactate	
	RLOD/HRP	LOD/HRP	RLOD/HRP	LOD/HRP
1mM glycolate	0	1.1	99.0	93.5
1 mM L-phenyllactate	1.1	6.1	100.0	102.1
10 mM D-lactate	0	0	99.7	99.8
10 mM L-α-hydroxycaproate	0	0	102.4	100.4
10 mM DL-α-hydroxybutyrate	2.5	3.2	97.8	94.1
10 mM pyruvate	0	0	100.1	100.2
10 mM L-α-hydroxyglutarate.	4.1	n.d.	103.5	n.d

n.d. - not determined

Tab. 5 Relative peak signal height in % of LOD modified electrodes in dependence on the coimmobilized peroxidase with respect to 100% in the absence of interferent.

interferent	HRP	FRP	MP-11	MP-8
	in the presence of 1mM L-lactat			
3.7 mM glutathione, reduced	91.9	87.8	41.4	64.0
1.15 mM ascorbate	−1268.0	−1905.0	−4921.7	−898.7
1.1 mM paracetamol	159.4	141.8	274.4	134.7
1.75 mM NaN_3	101.1	96.3	107.2	99.6
0.124 mM urate	97.7	111.8	106.0	98.8
L-cysteine: 0.12 mM	102.1	104.0	119.1	101.3
0.46 mM	100.1			
	in the absence of L-lactat			
3.7 mM glutathione, reduced	−7.3	−21.6	−39.0	−7.8
1.15 mM ascorbate	−1460.0	−2185.0	−5121.0	−1594.0
1.1 mM paracetamol	3.1	-8.2	−15.9	−5.9
1.75 mM NaN_3	0	0	23.1	0
0.124 mM urate	5.7	9.1	30.7	0
0.12 mM L-cysteine	9.7	15.2	10.5	0

Tab. 5 summarizes the influence of redox active species and peroxidase substrates on the L-lactate response for CPEs modified with different peroxidases. With respect to the following interferents: urate, L-cysteine, paracetamol, and ascorbic acid the HRP and ARP modified CPEs show a very similar selectivity pattern both in the absence and in the presence of L-lactate. With respect to NaN_3, urate, and cysteine, MP-8 modified CPEs show the highest selectivity. It seems probable that typical peroxidase substrates, *e.g.* phenol and aminobenzene derivatives and ascorbic acid strongly interfere at CPEs modified with peroxidases of the protoporphyrine IX type.

To investigate the oxygen influence on the FI response of the different EMCPEs totally and partially degassed lactate solutions were injected into the FIA set-up. No oxygen influence could be found in a confidence interval of $h = h_o \pm 0.05h_o$ with the peak height h_o measured after injection of air saturated 1 mM L-lactate solution. The high oxygen permeability through the PTFE tubing and the high oxygen concentration in the pasting liquid exclude any oxygen limitation.

Conclusion

Reagentless and mediatorless EMCPEs can be prepared from at least four different peroxidases, *e.g.* HRP, ARP, MP-8, and MP-11. Both RLOD and LOD can be coimmobilized with these peroxidases to prepare sensitive L-lactate selective CPEs.

The pH optimum of the resulting bienzyme modified CPEs are shifted significantly by selecting of the different peroxidases enabling an improved adaptation to the pH optimum of the activity of lactate oxidase and probably other oxidases.

Based on the adsorptive immobilization procedure the HRP and the ARP modified electrodes provides higher signal stabilities and lower background currents in comparison to the microperoxidase modified CPEs. The signal stability of all lactate oxidase/peroxidase

modified CPEs under FIA conditions can be considerably improved both with respect to the L-lactate and the H_2O_2 response by complexing of the peroxidases with polyethyleneimine before their adsorptive immobilization. If peroxidases of the protoporphyrine IX type have been used to prepare EMCPEs and other enzyme modified sensors, interferences by typical peroxidase substrates, *e.g.* phenols and aminobenzene as well as many of their derivatives have to be taken into consideration.

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