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THE EFFECT OF VARIOUS POLYELECTROLYTES ON ALCOHOL OXIDASE/HORSERADISH PEROXIDASE/FERROCENE MODIFIED CARBON PASTE ELECTRODES

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Two enzyme based alcohol biosensors were constructed by co-immobilizing alcohol oxidase and horseradish peroxidase into a carbon paste matrix. The effects of chemically modifying HRP, adding a mediator and different polyelectrolytes were examined, aiming at better operational stability when used in a flow injection system. The electrodes are characterized (sensitivity, detection limit, operational stability) when operated at an applied working potential of -50 mV vs. Ag/AgCl.

Introduction

Accurate determination of alcohol in low concentrations in complex matrixes has pushed the development of biosensors forward. There are several areas of

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interest, including the pulp-industry and food and environmental industry, where biosensors can be used for both process- and quality control. The most frequently studied types of enzymes for alcohol determination are alcohol oxidase (AOD) and alcohol dehydrogenase (ADH) [1-9]. The use alcohol dehydrogenase requires an addition of co-factor (NAD^+), complicating the construction of the biosensor. Alcohol oxidase, on the other hand, is dependant on the presence of molecular oxygen as co-factor, simplifying the design of the sensor, and was therefore preferred for the development of our sensor. Although it is possible to directly follow the conversion amperometrically (the decrease in oxygen or the production of hydrogen peroxide), this requires high overvoltages, decreasing the sensitivity and increasing the risk for bias reactions. A coupled enzyme system consisting of alcohol oxidase and horseradish peroxidase (HRP) allows us to work at a more suitable potential (-50 mV *vs.* Ag/AgCl) increasing the specificity of the sensor.

The sensitivity of the sensor can further be increased if a mediator is introduced into the carbon paste matrix, enhancing the electron transfer in the system. Alcohol oxidase is very specific for its co-substrate, and so far no mediator has been reported to work at a high reaction rate with the enzyme [10]. This introduces the possibility of mediating horseradish peroxidase without any short circuiting of the system, since the mediator will only enhance the electron transfer between HRP and the electrode itself. Several different mediators and techniques are possible including enzyme "wiring" [11], and in our case we choose to work with ferrocene.

Introducing the biosensor into a flow injection system will allow high throughput and repeatability along with the possibility of connecting a microdialysis sampling system [12] for on-line measurements. Using the biosensor in industrial applications puts high demands on the operational stability. Several factors influence the operational stability, such as enzyme manufactures, the addition of soluble mediators, enzyme modification and the addition of polyeletrolytes. Concentrating on the polyelectrolytes, we have examined the effects of adding polyethylenimine (PEI), poly-L-lysine and a poly (ester) sulfonic acid (EAQ 29D) into the carbon paste matrix.

Experimental

Chemicals

AOD (EC 1.1.3.13) from Hansenula polymorfa obtained as a buffered solution (100 mM MOPS, pH 7.9) with an activity of 450 U ml^{-1} was purchased from Leeds Biochemicals, Leeds, UK. HRP (EC 1.11.1.7) was obtained as lyophilised powder with a minimum activity of 1000 U mg^{-1} from Boehringer-Mannheim, Mannheim, Germany. Carbon powder (Cat. No. 50870), paraffin oil (Cat. No.

76235) and ferrocene (Cat. No. 46260) were purchased from Fluka, Buchs, Switzerland. Polyethylenimine (PEI) 50 % aqueous solution (Cat. No. P-3143) and poly-L-lysine (Cat. No. P-2636) were obtained from Sigma, St. Louis, MO, USA. The polyanion (EAQ 29D) 30 % aqueous solution, was purchased from Eastman Chemical Products, Kingsport, TN, USA and sodium periodate ($NaIO_4$) 99 %, (Cat. No. 19.838.50) from Janssen, Chimica, Geel, Belgium. Ethanol, 99.5 %, was purchased from Kemetyl, Stockholm, Sweden. All other chemicals were from Merck, Darmstadt, Germany and solutions were prepared using water produced in a Milli-Q system from Millipore, Bedford, MA, USA.

Electrode Preparation

A) 4.0 mg HRP were dissolved in 400 µl 0.05 M phosphate buffer (pH 8.0) and added to 200 mg carbon powder and mixed for 30 min at 4 °C. Next 400 µl AOD were added, followed by 1 hour of mixing at 4 °C. The enzymatic powder was then left to dry for 4 h in a vacuum jet desiccator. After drying, paraffin oil was added to yield a paste containing 24.24 % (w/w) oil. The paste was mixed by hand for 15 min. in a mortar.

B) The paste was prepared as (A) with the difference that the paraffin oil contained 1.32 % (w/w) ferrocene, resulting in a mediated paste containing 0.32 % (w/w) ferrocene.

C) Chemical oxidation of the carbohydrate "shell" of HRP was performed by dissolving 4.0 mg HRP in 1.25 ml H_2O containing 1.6 mg l^{-1} $NaIO_4$ and mixing the solution for 20 min at room temperature [13]. In the following step the solution was centrifuged through a Centricon-30 concentrator (Amicon Inc., Beverly, MA, USA) with a cut-off of 30 000 MW (Cat. No. 4208) to remove the sodium periodate. The enzyme was then dissolved in 400 µl 0.05 M phosphate buffer (pH 8.0). Thereafter the paste was prepared according to (B).

D) The HRP was modified according to (C) and added to 200 mg carbon powder and mixed for 30 min at 4 °C. Next 400 µl AOD were added together with either 400 µl of PEI solution (prepared by mixing 100 mg of 50 % PEI with 5 ml H_2O) or 400 µl of poly-L-lysine-solution (prepared by mixing 8.0 mg poly-L-lysine with 400 µl of PB (0.05 M, pH 8)), followed by 1 h of mixing at 4 °C. The enzymatic powder was then left to dry for 4 h in a vacuum jet desiccator. After drying, paraffin oil (containing 1.32 % (w/w) ferrocene) was added to yield a paste containing 24.24 % (w/w) oil. The paste was mixed by hand for 15 min in a mortar.

E) A PEI containing paste was prepared according to (D) with an addition of 80 µl EAQ 29D solution (prepared by mixing 10 µl EAQ 29D (30 %) with 740 µl H_2O), added together with the PEI-solution.

The electrodes were prepared by filling plastic cartridges with plain unmodified paste, leaving 2–3 mm empty at the top to be filled with modified paste. The inner diameter of the cartridge was 2.9 mm, resulting in a surface area of 6.605 mm^2. A metal screw, fabricated with the same diameter as the cartridge was used to push the paste out of the cartridge.

All flow injection experiments were carried out with 0.1 M phosphate buffer (PB), pH 7.0, as the mobile phase.

Instrumentation

The modified electrodes were inserted into a three electrode flow-through cell of wall-jet type [14]. The cell consisted of an Ag/AgCl reference electrode and a platinum counter electrode (see Fig. 1). The cell was inserted into a flow injection system consisting of a HPLC pump (LKB, Bromma, Sweden, mod. 2150) pumping at a flow rate of 0.8 ml min^{-1}, a second pump (Gilson, Villier-le-Bel, France, mod. Minipuls 2) used for filling the injection loop, and a pneumatically controlled injection valve (Cheminert, Cotati, CA, USA, type SVA) with a 50 µl injection loop (see Fig. 2). A potentiostat (Zäta Elektronik, Lund, Sweden) was connected to the cell and the current was recorded on a recorder (Kipp & Zonen, Delft, The Netherlands, mod. BD11). The operational stability experiments were performed using an Automated Sample Injection Analyzer (Ismatec, Glattburg-Zürich, Switzerland) with a 50 µl injection loop.

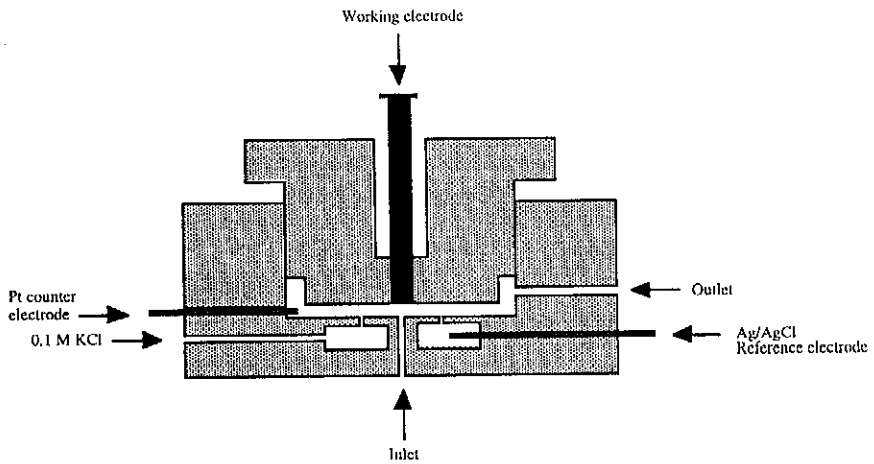

Fig. 1 Schematic drawing of the flow-through wall-jet cell

Results and Discussion

The basic reaction sequence of an AOD/HRP-based biosensor is illustrated below. In the first step ethanol is oxidized by AOD, producing acetaldehyde and hydrogen peroxide (Reaction 1).

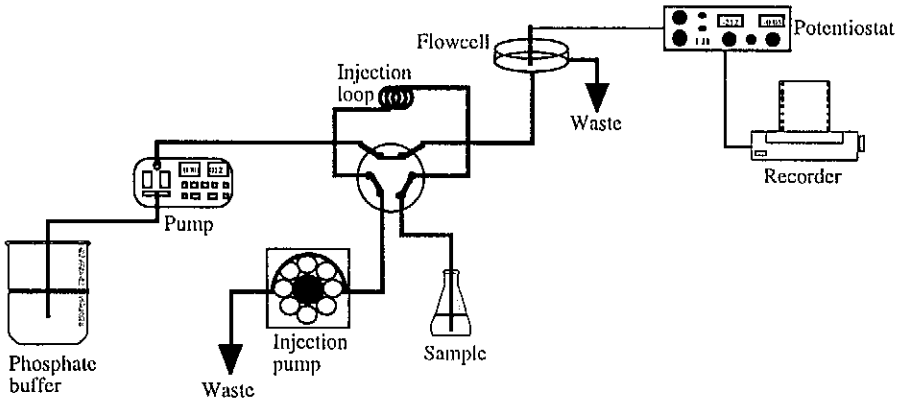

Fig. 2 Schematic drawing of the flow-injection system

$$\text{ethanol} + O_2 \xrightarrow{\text{AOD}} \text{acetaldehyde} + H_2O_2 \qquad (1)$$

In the following step the produced H_2O_2 is reduced to water in a single two-electron process by HRP, resulting in the oxidized form of the enzyme (Reaction 2). The oxidized form is then re-reduced to its native form in two one-electron transfer steps (Reactions 3 and 4).

$$H_2O_2 + HRP_{native}(+3) \rightarrow H_2O + HRP_{ox}/\text{compound I}(+5) \qquad (2)$$

$$\text{compound I}(+5) + e^- + H^+ \rightarrow \text{compound II}(+4) \qquad (3)$$

$$\text{compound II}(+4) + e^- + H^+ \rightarrow HRP_{native}(+3) + H_2O \qquad (4)$$

The re-reduction of compound I and II can depend on either direct electron transfer, when the enzyme accepts electrons directly from the electrode, or on a mediated electron transfer where the enzyme accepts the electrons from a suitable mediator. The mediator can then be re-reduced by accepting electrons from the electrode if the applied potential is more negative than the formal potential of the redox-mediator.

Although it is possible to construct an ethanol carbon paste biosensor based on AOD and HRP without mediators or polyelectrolytes, such a sensor

provides low sensitivity and a poor detection limit [7]. This was also our experience and, as shown in Fig. 3, the sensor based on this approach (Type A) gave the lowest sensitivity. Introducing a mediator to this type of electrode (Type B) increased the sensitivity slightly but it was still too low to give accurate results when used as an analytical tool (Fig. 3).

Decreasing the distance between the enzymes in the paste will increase the sensitivity since the hydrogen peroxide will have shorter distance to diffuse, which will result in a higher trapping efficiency of H_2O_2. One possibility of achieving increased contact between the enzymes is to chemically modify HRP

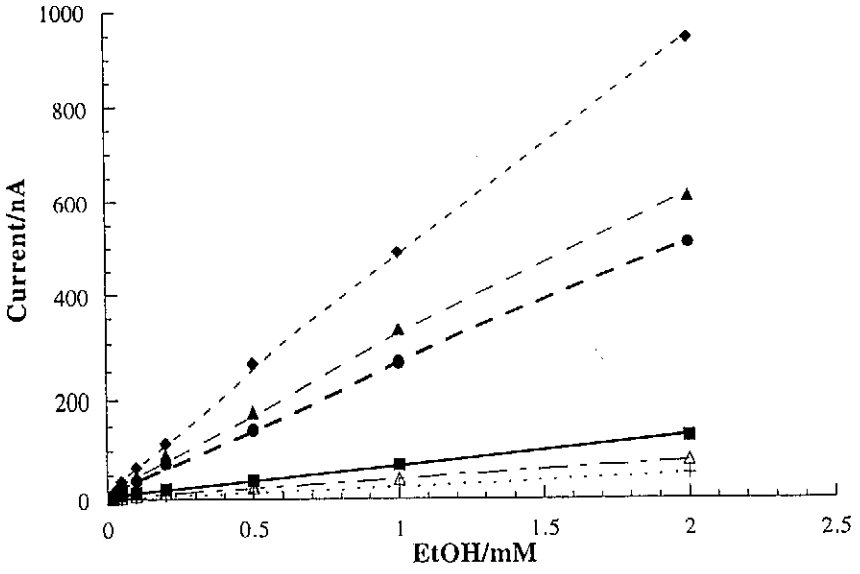

Fig. 3 Calibration curves (0.02-2.00 mM ethanol) for all electrode configurations, (+) Type A, (Δ) Type B, (■) Type C, (●) Type D (PEI), (▲) Type D (poly-L-lysine), and (♦) Type E, registered in the FI-system (flow rate 0.8 ml min^{-1}, E_{appl} = -50 mV *vs.* Ag/AgCl)

[13]. By oxidizing the carbohydrate "shell" on the enzyme, resulting in more reactive groups such as aldehydes on the enzyme surface, it is possible to bind it to AOD and create closer contact between the enzymes. This also resulted in a further increased sensitivity (Fig. 3, Type C), although it was still very low.

AOD is a large (600 000 Da) negatively charged enzyme containing eight subunits [15]. By introducing some positively charged polyelectrolytes into the paste it should be possible to increase the stability of the enzyme. It has also previously been shown that the polyelectrolytes increase the sensitivity of a carbon paste sensor, possibly as a result of increasing the turn over rate of the enzyme. PEI is a widely used stabilizer but others have been used as well, such as polylysine, polyarginine, EAQ etc. [16,17]. In our case three electrode

configurations were examined, the addition of PEI or poly-L-lysine (Type D) and a combination of PEI and EAQ (Type E). As illustrated in Fig. 3 all three types showed a high increase in sensitivity compared to the electrodes without polyelectrolytes. The electrode with the combination of PEI and the negatively charged EAQ has a very high sensitivity indicating that a combination of charges has a very positive effect on the sensors performance. It is possible that some kind of network of charges is created, stabilizing the enzyme and increasing the electron transfer.

Table I The detection limit was calculated as twice signal to noise and the sensitivity was calculated from the calibration curves linear regression

Electrode configuration	Sensitivity, nA mM^{-1} cm^2	Detection limit, mM
Type A	0.385	0.032
Type B	0.580	0.017
Type C	0.933	0.007
Type D (PEI)	3.808	0.005
Type D (poly-L-lysine)	4.560	0.005
Type E	7.094	0.003

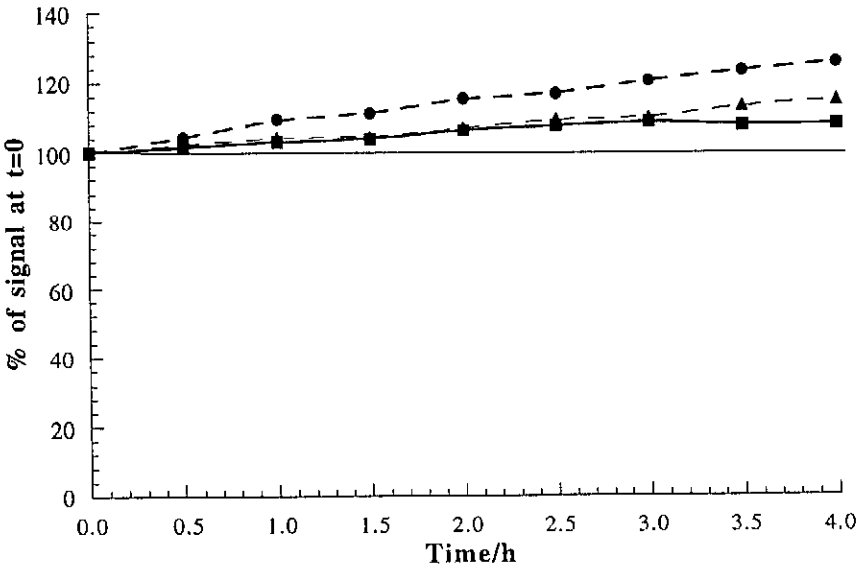

Fig. 4 Operational stability examined for the modified carbon pastes containing polyelectrolytes during 4 h of continuous use in the FI-system, (■) Type D (PEI), (●) Type D (poly-L-lysine) and (▲) Type E

Table I summarises the sensitivity and detection limits of the investigated electrode configurations.

The operational stability, being an important factor when the sensors are used in a flow injection system, was investigated for a 4 hour period for the electrodes containing the polyelectrolytes (see Fig. 4). Surprisingly, there is an increase in response signal with time and it can somewhat be explained by temperature differences in the laboratory, but the high increase for the electrode with poly-L-lysine indicates that there is a possible activation of the surface or the enzymes when the electrode is in use. This phenomena requires further investigations to be explained and are beyond the scope of this paper.

Conclusion

The addition of polyelectrolytes to the paste improves both the sensitivity and the detection limit at least by a factor of 10. It has been shown that a combination of both positively and negatively charged electrolytes increases the sensitivity further and gives the electrode an operational stability that will make it useful for incorporation into an on-line monitoring system. This electrode configuration had a sensitivity of S = 7.094 nA mM^{-1} cm^2 and a detection limit of 0.003 mM (S/N = 2) and a linear range up to 2.0 mM of ethanol.

Acknowledgements

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