

Direct detection of formaldehyde in air by a novel NAD⁺- and glutathione-independent formaldehyde dehydrogenase-based biosensor

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Received 30 July 2007; received in revised form 29 November 2007; accepted 7 December 2007

Available online 23 December 2007

Abstract

An amperometric enzyme-based sensor-system for the direct detection of formaldehyde in air is under investigation. The biosensor is based on a native bacterial NAD⁺- and glutathione-independent formaldehyde dehydrogenase as biorecognition element. The enzyme was isolated from *Hyphomicrobium zavarzinii* strain ZV 580, grown on methylamine hydrochloride in a fed-batch process. The sensor depends on the enzymatic conversion of the analyte to formic acid. Released electrons are detected in an amperometric measurement at 0.2 V vs. Ag/AgCl reference electrode by means of a redox-mediator. To optimize the sensing device, Ca²⁺ and pyrroloquinoline quinone (PQQ) were added to the buffer solution as reconstititional substances.

At this stage, the sensor shows linear response in the tested ppm-range with a sensitivity of 0.39 μ A/ppm. The signal is highly reproducible with respect to sensitivity and base line signal. Reproducibility of sensitivity is more than 90% within the same bacterial batch and even when enzyme of different bacterial batches is used.

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Keywords: Enzyme sensor; Gas-phase; Gas sensor; Amperometric biosensor; Dye-linked formaldehyde dehydrogenase; *Hyphomicrobium zavarzinii*

1. Introduction

In spite of their high sensitivity and selectivity the use of biosensors for industrial or medical analysis is still restricted. Enzyme-based sensor-systems still suffer from their low long-time stability often provoked by the low stability of their biological components [1]. They are also often restricted to analytes in solvent or fluidic analysis-systems, because an appropriate medium (solvent or gel) adjusted to the used enzymatic system [2] is needed, to gain optimum activity and stability of the enzyme and other biological components. Thus, for gas phase analysis additional sampling and accumulation steps have to be incorporated prior to the real measurement, showing a few drawbacks such as high cost for technical equipment, longer sampling time and sometimes reduction of measuring-range.

The enzyme-based sensor-system investigated here is designed to avoid some of the difficulties mentioned above. In contrast to existing systems [3–5], the amperometric biosensor detects the analyte formaldehyde directly from the gas phase without prior accumulation or sampling steps. So, it can be used as an online detection system to monitor the formaldehyde concentration in ambient air.

Unlike other biosensors detecting formaldehyde, which often use commercially available formaldehyde dehydrogenase from *P. putida* [6,7], or another NAD⁺- and/or glutathione-dependent formaldehyde dehydrogenase [8], the novel biosensor uses a dye-linked formaldehyde dehydrogenase from *H. zavarzinii* strain ZV 580 as biorecognition element. This enzyme catalyses the conversion of the analyte without the need of NAD⁺, a cofactor which is very well known in the literature [9] for its instability in different buffers, its high over potential for direct re-oxidation, and a proposed radical reaction mechanisms during re-oxidation [10]. Therefore, a NAD-independent system is expected to show a better long-term stability than the available systems.

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A dye-linked formaldehyde dehydrogenase (DL-FDH) from *H. zavarzinii* strain ZV 580 was earlier described by Klein et al. [11] and further characterized by Schwartz et al. [12]. Also not yet verified, pyrroloquinoline quinone (PQQ) may be the bound cofactor, and it is generally assumed that Ca^{2+} ions are required for the stabilization of the tetrameric structure of DL-FDH and for its optimal functionality [13].

In this study, the sensitivity of partially purified DL-FDH from *H. zavarzinii* strain ZV 580 on formaldehyde in an amperometric biosensor system, the cross-sensitivity and the effect of adding Ca^{2+} ions and PQQ on the sensor signal are investigated.

2. Materials and methods

2.1. Chemicals

1,2-Naphthoquinone-4-sulfonic acid (sodium salt, approx. 97%, NQS) was purchased from Sigma, NAD^+ (free acid, approx. 95%) from Merck. Formaldehyde sample solutions were prepared from a 36.5% stock solution (formalin solution, Riedel-de-Haen), containing 10% methanol as stabilizer, by dilution. All other chemicals were of analytical grade. Distilled water was used to prepare standard and buffer solutions.

2.2. Organism and growth

The α -proteobacterium *H. zavarzinii* strain ZV 580 was cultivated as described previously [14]. The strain *H. zavarzinii* ZV 580 was from the collection of the “Institut für Allgemeine Mikrobiologie” (University of Kiel, Germany) and a generous gift from Dr. Vorholt (INRA/CNRS, Castanet-Tolosan, France).

Briefly, bacteria were grown in a mineral medium in a fully automated fed-batch process in a NLF22 bioreactor from Bioengineering (Wald, Switzerland) with 10 mM methyamine hydrochloride supplemented to the culture medium as C-source. The mineral medium contained 10 mM K_2HPO_4 , 14 mM Na_2HPO_4 , 10 mM MgSO_4 , 15 mM $(\text{NH}_4)_2\text{SO}_4$, 4 μM FeSO_4 , 1 μM ZnSO_4 , 10 μM CaCl_2 , 1 μM CuSO_4 , 1 μM CoCl_2 , 186 μM $(\text{NH}_4)_6\text{Mo}_7\text{O}_{24}$, 5 μM MnCl_2 and 4 μM EDTA and was kept at a pH of 6.9 ± 0.1 .

2.3. Preparation of active DL-FDH

To receive active DL-FDH for application in the biosensor device, cell-free extract from the cultivated *H. zavarzinii* was prepared as described previously [14].

In short, the cell lysate was first saturated to 40% with ammonium sulfate, stirred for 60 min at 4 °C and the resulting precipitate was discarded. Subsequently the ammonium sulfate concentration was raised to 60% and the precipitate obtained after 60 min stirring at 4 °C (later called P60 or P60-fraction) containing the DL-FDH was collected by centrifugation and resuspended in a minimal volume of buffer (50 mM potassium phosphate buffer pH 8.0). The P60 sample was desalted using a “Zeba™ desalt” spin column (Pierce, Bonn, Germany) and the final enzyme concentration was determined using a micro-BCA

Table 1

Formaldehyde concentration in the aqueous phase and the corresponding equilibrium gas phase concentrations at 20 °C according to the equation given by Dong and Dasgupta [15]

Concentration of CH_2O in the aqueous phase (mM)	Concentration of CH_2O in the gas phase (vppm)
2.62	0.5
11.69	2
31.4	5
66.4	10
103	15

protein assay (Pierce, Bonn, Germany), both according to the manufacturer’s instructions.

2.4. Sensor set-up

The sensor is based on the enzymatic conversion of the analyte by DL-FDH from *H. zavarzinii* strain ZV 580 and subsequent electrochemical detection of released electrons with the aid of 1,2-naphthoquinone-4-sulfonic acid (NQS) as mediator (Fig. 1). The flux of electrons was recorded in an amperometric measurement at +200 mV vs. Ag/AgCl reference (3 M KCl, 210 mV vs. NHE) using a potentiostat (PGSTAT 12, Eco Chemie, The Netherlands).

The sensor device consists of a 3-electrode configuration with disks of woven graphite gauze (Alfa Aesar, $\varnothing = 15$ mm) as working and counter electrode included in a plastic housing (Fig. 2). Both electrodes were contacted with Pt-wire ($\varnothing = 0.2$ mm). The gas diffuses into the liquid phase via a 15 mm diameter Teflon membrane (FALP02500, Millipore, France). Loss of enzyme is restricted by a dialyses membrane with a MWCO of 12,000–14,000 Da (Medicell International, London, Great Britain).

Gaseous formaldehyde samples were collected from the head space above aqueous solutions of known concentration. The CH_2O concentration in the gas phase above the solution was calculated according to the equation given by Dong and Dasgupta [15] as shown in Table 1.

The buffer within the sensor contained 0.1 M KCl with 80 mM phosphate at pH 8. The mediator NQS was added in a concentration of 5 mM to the phosphate buffer. Experiments were conducted with varying formaldehyde concentrations as stated in Table 1 at room temperature and the sensing device was freshly prepared before each measurement.

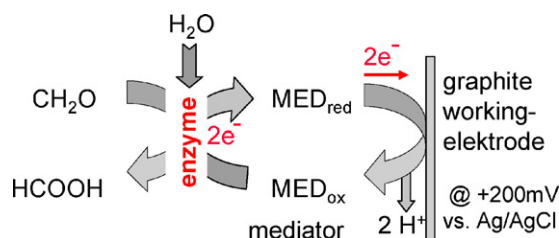


Fig. 1. Mechanism of the measurement of released electrons in the enzyme catalyzed conversion step of formaldehyde to formic acid.

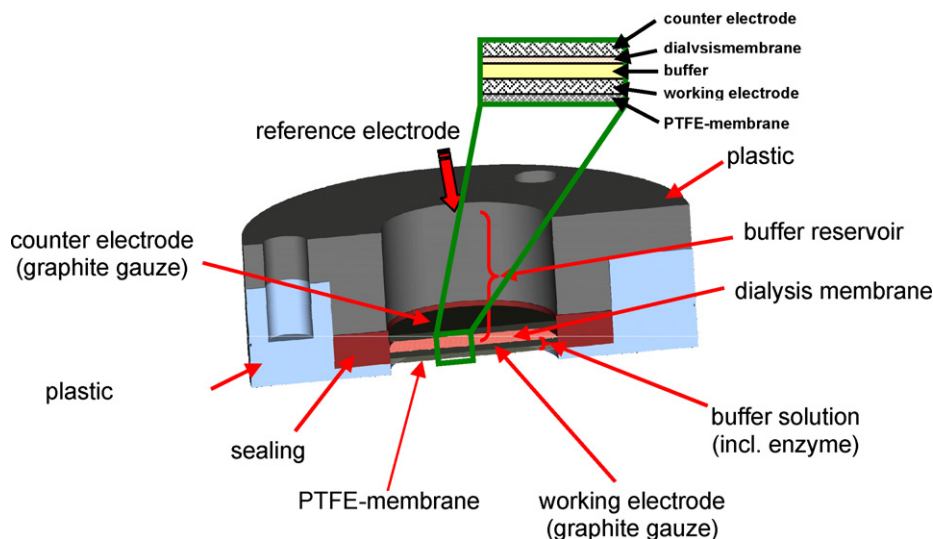


Fig. 2. Sensor set-up: plastic housing with different membranes and electrodes for the detection of the gaseous analyte formaldehyde.

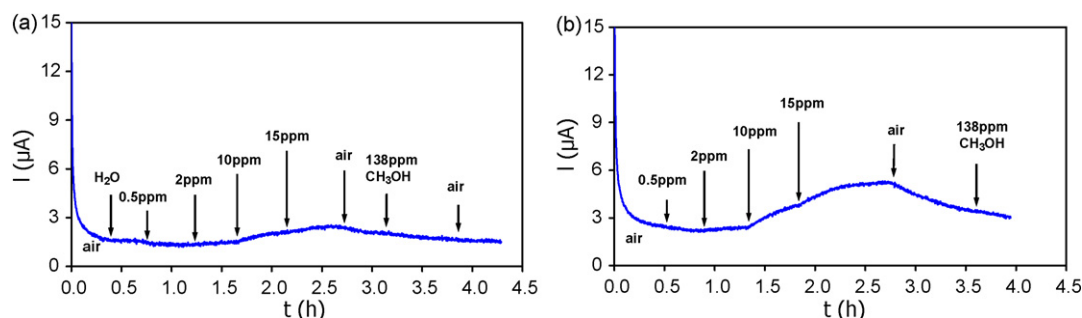


Fig. 3. Sensitivity enhancement by quantitative increase of the enzyme load in use. (a) 300 μg protein included in P60-fraction in 200 μl buffer, (b) 600 μg protein in P60-fraction in 200 μl buffer. Sensor response to formaldehyde and methanol vapor above aqueous sample solutions. Potential applied: +200 mV vs. Ag/AgCl. Buffer composition: 0.1 M KCl, 80 mM KH_2PO_4 , pH 8, 5 mM NQS. $c(\text{CH}_2\text{O}) = 0.5\text{--}15$ vppm, $c(\text{CH}_3\text{OH}) = 30$ mM (138 vppm in the gas phase [16,17]), both as indicated.

3. Results and discussion

3.1. Sensor characteristics

In order to optimize the sensitivity of the sensor, two different possibilities were examined: first, the enzyme load was gradually increased by enhancing the amount of P60-fraction in use. For sensor tests a P60-fraction with a final enzyme concentration, determined from micro-BCA protein assay, of 18 mg/ml was used with a specific activity of 0.023 U/mg protein (one unit is defined as the amount of enzyme required for reduction of 1 μmol of dichlorophenolindophenol (DCPIP) per minute). In detail, the enzyme load was increased from 300 μg to 1200 μg of protein included in the P60-fraction.

The sensitivity was enhanced by doubling the enzyme load in use from 76 nA/ppm to 200 nA/ppm when 300 μg or 600 μg protein in P60-fraction were tested, respectively (Figs. 3a, b and 4). Obviously, enzyme saturation is not reached for the amount of formaldehyde that has to be converted and the reaction is quantitatively limited by the enzymatic conversion rate. So in a range, current is also a function of the enzyme load not only of the concentration of the analyte.

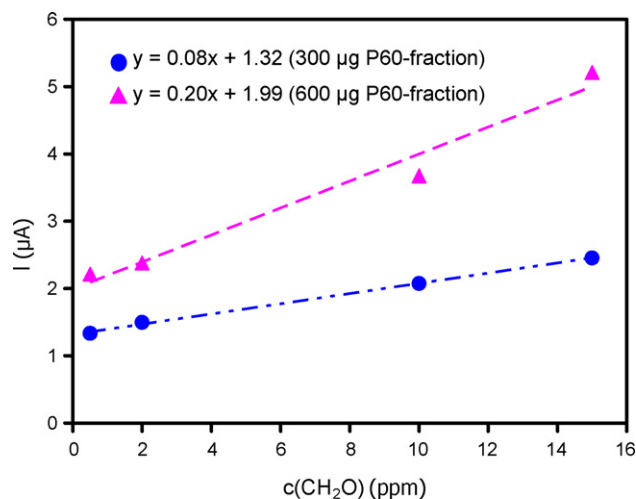


Fig. 4. Characteristic response curve of a sensor with an enzyme load of (●) 300 μg protein included in P60-fraction and (▲) 600 μg protein of P60-fraction in 200 μl buffer. Potential applied: +200 mV vs. Ag/AgCl. Buffer composition: 0.1 M KCl, 80 mM KH_2PO_4 , pH 8, 5 mM NQS.

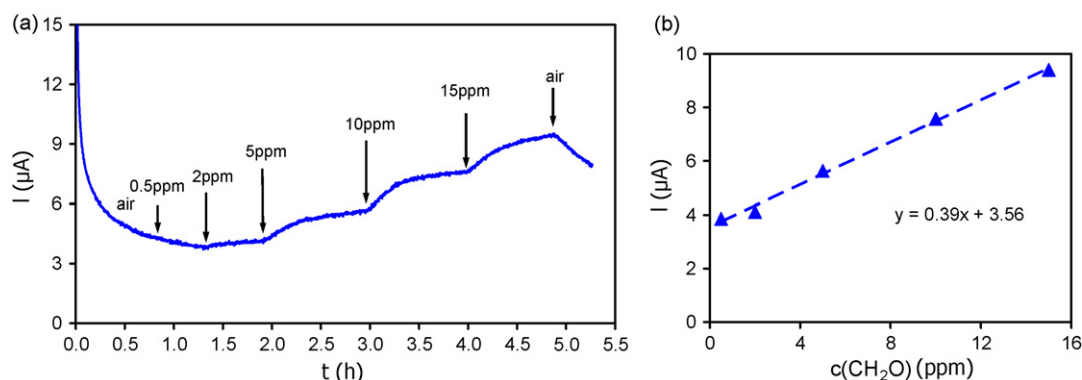


Fig. 5. (a) Response of the sensor to the vapor above aqueous sample solutions of formaldehyde and methanol when an enzyme load of 1200 μg protein in P60-fraction is used and PQQ and Ca^{2+} was added. Potential applied: +200 mV vs. Ag/AgCl. Buffer composition: 0.1 M KCl, 80 mM KH_2PO_4 , pH 8, 5 mM NQS, 1 μM PQQ, 50 μM CaCl_2 . Enzyme loading: 1200 μg protein in P60-fraction in 200 μl buffer. (b) Linear response curve of the sensor in the tested range of 0.5–15 vppm CH_2O .

Another possibility to enhance the sensor sensitivity is to improve the activity of the enzyme itself by adding PQQ (1 μM) and Ca^{2+} ions by means of 50 μM CaCl_2 to the buffer solution as previously reported in Ref. [14]. This so called reconstitution of the active form of the enzyme increases the sensor sensitivity from 200 nA/ppm to 270 nA/ppm in the case of 600 μg un-reconstituted and reconstituted protein in P60-fraction, respectively, i.e. approximately 25% higher sensitivity (results not shown). That is again due to the fact that higher enzyme activity leads to higher enzymatic conversion rates, resulting in a higher sensor signal.

At present, highest sensitivity is realized by adding PQQ and Ca^{2+} to 1200 μg protein in P60-fraction and implementing this for biorecognition. A characteristic signal is shown in Fig. 5(a, b) with a sensitivity of 390 nA/ppm, a t_{90} -time of about 15 min and a linear response curve in the tested range.

3.2. Selectivity of the sensing device

To evaluate the selectivity of the sensing device, interference from methanol and the influence of water vapor were of special interest.

Methanol is used in a concentration of 10% as a stabilizing agent in the formalin solution, from which gaseous formaldehyde samples were prepared. Furthermore, methanol is also a possible C-source for *H. zavarzinii* growth, so that the enzyme preparation may contain a methanol metabolizing enzyme, too.

Aqueous solutions of methanol in the range of 3–60 mM were prepared and tested, to rule out the possibility that methanol instead of formaldehyde is responsible for the sensor signal or that there are any interferences by methanol. For comparison, an aqueous solution of 103 mM CH_2O , corresponding to 15 vppm CH_2O in the gas phase, contains about 19 mM methanol. So, the range mentioned above exceeds the amount of methanol included in the formaldehyde samples by far.

Gaseous methanol for interference tests were again sampled above the head space of the mentioned aqueous solutions. Gas phase concentrations as shown in Table 2 were calculated by Henry's law with a Henry's law constant given by Sander [16] and Snider and Dawson [17].

Table 2

Methanol concentration in the gas phase above aqueous methanol sample solutions calculated by an Henry's law constant at standard conditions (298.15 K) given by Sander [16] and Snider and Dawson [17]

Concentration of CH_3OH in the aqueous phase (mM)	Concentration of CH_3OH in the gas phase (vppm)
3	13.8
30	138
60	276

The sensor showed no sensitivity to gaseous methanol in the tested range, at all (Fig. 6) so that it can be stated, that there is no influence of methanol on the sensor signal and that the signal does not result from any conversion of methanol included in the test samples.

Due to the fact that all gaseous samples are derived from aqueous solutions, interferences from water vapor were also evaluated but it was found that water vapor did not affect the base signal of the sensor (results not shown).

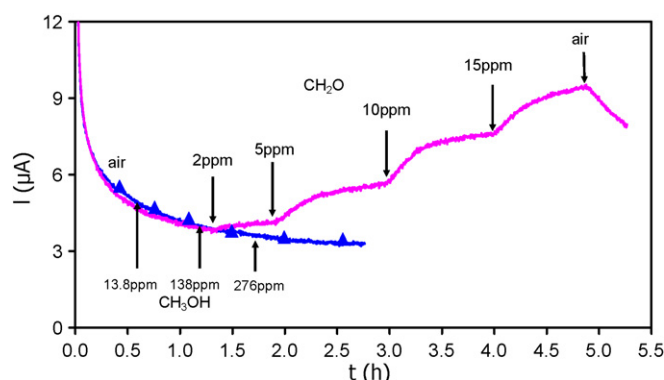


Fig. 6. Selectivity data of the sensing device. Methanol was used as possible interfering substance in the range of 3–60 mM in solution. ($\blacktriangle$) Gaseous methanol samples generated by aqueous solutions; (—) gaseous formaldehyde samples. Potential applied: +200 mV vs. Ag/AgCl. Buffer composition: 0.1 M KCl, 80 mM KH_2PO_4 , pH 8, 5 mM NQS, 1 μM PQQ, 50 μM CaCl_2 . Data received from two freshly prepared sensors with an enzyme load of 1200 μg protein in P60-fraction in 200 μl buffer.

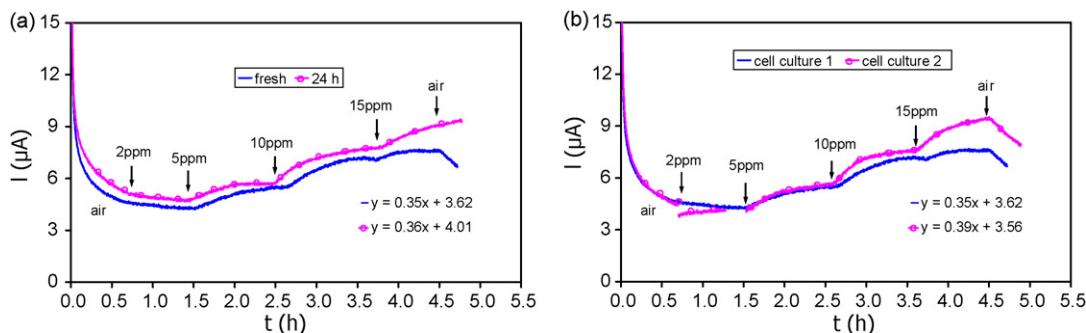


Fig. 7. Reproducibility of the sensor. Different 1200 μg P60-fraction enzyme samples in 200 μl buffer are used. (a) Effect of deterioration during storage for 24 h at 4 °C: (—) freshly prepared enzyme samples as biorecognition element; (○) enzyme stored at 4 °C for 24 h. (b) Enzyme samples of diverse *Hyphomicrobium zavarzinii* batch cultures: (—) culture 1; (○) culture 2. Potential applied: +200 mV vs. Ag/AgCl. Buffer composition: 0.1 M KCl, 80 mM KH₂PO₄, pH 8, 5 mM NQS, 1 μM PQQ, 50 μM CaCl₂. $c(\text{CH}_2\text{O}) = 2\text{--}15$ vppm.

3.3. Reproducibility of the sensor signal

Reproducibility of the sensor signal was especially evaluated with regard to deterioration of the enzyme sample and influences of microbial enzyme production.

Therefore, enzyme samples isolated from *H. zavarzinii* grown in different bioreactor batches were examined. Deterioration was reviewed after the enzyme sample was kept at 4 °C for 24 h.

The signal was highly reproducible when enzyme of the same bacterial batch was used and the influence of deterioration during storage was quite low. After 24 h at 4 °C the reproducibility of the sensor signal is about 98.5% related to freshly prepared enzyme (Fig. 7a, 350 nA/ppm and 360 nA/ppm, respectively). Even when enzyme samples isolated from *H. zavarzinii* cells grown in different bioreactor batches were used, the signal was very repeatable. As can be seen from Fig. 7b, the sensitivity of the sensing device is altered from 350 nA/ppm to 390 nA/ppm for two different cell cultures, meaning a reproducibility of about 92%. In this case the linear range of the sensor prepared with cell culture 1 from 0 vppm up to 10 vppm was taken into account.

Reproducibility data after storage are quite promising. After optimizing the storage procedure there will be hardly any disadvantages of producing the enzyme samples in greater amount in one bioreactor process and store them for later use.

3.4. Cofactor mediated biosensor

In order to reduce the number of components involved in the reaction chain, PQQ which is a possible mediator for dehydrogenase enzymes [18,19], was used as mediating cofactor and reconstitution element simultaneously. The cofactor was used in concentrations of 1 μM and 10 μM , added to the buffer solution.

The sensor shows a formaldehyde concentration dependent signal, when 1 μM PQQ is used. The sensitivity of the sensor is 140 nA/ppm meaning about 35% of the sensitivity of a reconstituted sensor with an enzyme load of 1200 μg protein in P60-fraction (cp. Fig. 5, sensitivity 390 nA/ppm). Increasing the PQQ concentration to 10 μM does not lead to higher sensitivity of the sensor (results not shown).

Without the addition of any mediator or of PQQ, a very low electron transfer rate to the electrode was detected (Fig. 8). A possible reason for this low current are not yet identified redox-active substances in the protein fraction that can be converted at the electrode producing an amperometric signal. It is also possible, that the enzyme itself is directly re-oxidized at the electrode surface. For this possibility the sensitivity decline or may be loss at 10 vppm would indicate an electron transfer limitation for higher analyte concentrations.

Further investigations have to be done to clarify this phenomenon.

3.5. Complexation of Ca²⁺

A quasi-negative control was prepared by complexation of the structurally important Ca²⁺ by the addition of 1 mM EDTA to the buffer solution. In that case, only 1 μM PQQ was added to the buffer solution without CaCl₂.

The sensor response to formaldehyde is almost completely lost when Ca²⁺ is complexed by EDTA (Fig. 9, time gaps result from inconsistent measurement periods of the two experiments).

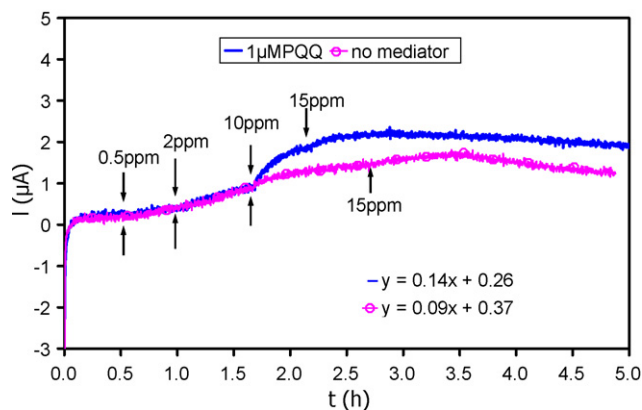


Fig. 8. Sensor signal for a PQQ mediated biosensor and a biosensor where no mediator was added, enzyme load: 1200 μg protein in P60-fraction in 200 μl buffer. (—) 1 μM PQQ is added to a buffer solution (0.1 M KCl, 80 mM KH₂PO₄, 55 μM CaCl₂, pH 8) (○) no mediator is added to the buffer solution (0.1 M KCl, 80 mM KH₂PO₄, 50 μM CaCl₂, pH 8). Potential applied: +200 mV vs. Ag/AgCl. $c(\text{CH}_2\text{O}) = 0.5\text{--}15$ vppm.

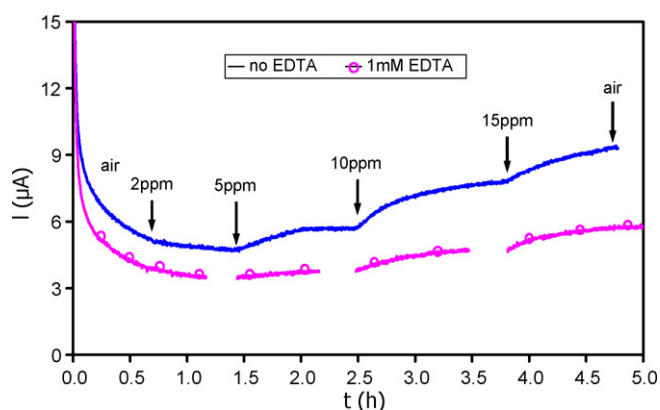


Fig. 9. Complexation of Ca^{2+} by EDTA: comparison of a sensor signal characteristic for a (—) PQQ and CaCl_2 reconstituted enzyme sample (buffer: 0.1 M KCl, 80 mM KH_2PO_4 , pH 8, 5 mM NQS, 1 μM PQQ, 50 μM CaCl_2) and (---) an enzyme sample where Ca^{2+} is complexed by the addition of 1 mM EDTA to the buffer solution (buffer: 0.1 M KCl, 80 mM KH_2PO_4 , pH 8, 5 mM NQS, 1 μM PQQ). Potential applied: +200 mV vs. Ag/AgCl. Enzyme load: 1200 μg protein in P60-fraction in 200 μl buffer. $c(\text{CH}_2\text{O}) = 2\text{--}15$ vppm.

As it was corroborated by the loss of sensitivity through complexation, Ca^{2+} plays an important role in the active centre of DL-FDH and for the sensing purpose.

It is likely that Ca^{2+} already embedded in the enzyme structure cannot be complexed by the added EDTA in solution and so a negligible amount of enzyme is still possible to convert formaldehyde causing the marginal signal shown in Fig. 9.

4. Conclusion and perspectives

In the studies presented above, native DL-FDH isolated from *H. zavarzinii* strain ZV 580 was successfully implemented in a sensing device. Using the DL-FDH it was possible to detect gaseous formaldehyde with a dehydrogenase enzyme without the addition of the unstable, unbound cofactor NAD^+ directly from the gas phase. With the elimination of NAD^+ it is much more promising to obtain a sensor device with higher long-term stability.

Further on, it was demonstrated that the detection of gaseous formaldehyde by DL-FDH is selective against methanol and that water vapor does not influence the measurement result at the air saturation point at room temperature.

Reproducibility of the measured signal was over 90% when the sensitivities of deteriorated enzyme samples and even of enzyme samples isolated from different *H. zavarzinii* cultures were compared to freshly prepared protein fractions.

Reconstitution with PQQ and Ca^{2+} improved the sensitivity of the device and showed the importance of these substances for the activity of the enzyme.

First experiments with a cofactor mediated reaction chain were very promising. Using a PQQ-functionalized electrode, much more effective electrical contact between enzyme and electrode might be possible. Anyway, the elimination of another additional substance, in this case the additional mediator NQS, will again enhance the feasibility to get a more stable sensor device.

For this, further work is under investigation to determine the dependence of the sensor stability from different factors and to evaluate the benefit of the formerly stated improvements.

Acknowledgement

Financial support by the *Deutsche Forschungsgemeinschaft* (Ha 4424/1-1).

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