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Development of a bifunctional sensor using haptenized acetylcholinesterase and application for the detection of cocaine and organophosphates

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ABSTRACT

We developed a dual piezoelectric/amperometric sensor for the detection of two unrelated analytes in one experiment that uses propidium to anchor acetylcholinesterases (AChE) at the surface. This mass-sensitive sensor does not only allow the examination of the interaction between AChE and the modified surface but also the detection of in situ inhibition of the surface-bound AChE.

Here we describe the application of the propidium-based sensor in combination with a modified AChE. For this reason the cocaine derivative benzoylecgonine (BZE) was coupled via a 10 Å long hydrophilic linker – 1,8-diamino-3,4-dioxaoctane – to carboxylic groups of the AChE after EDC/NHS activation. Thus the modified AChE (BZE–AChE) possesses an additional recognition element besides the inhibitor binding site. After the deposition of BZE–AChE on the sensor surface the binding of an anti-BZE-antibody to the BZE–AChE can be monitored. This makes it possible to determine two analytes – cocaine and organophosphate – in one experiment by measuring antibody binding and decrease in enzymatic activity, respectively. Furthermore it was also shown that other cocaine-binding enzymes, e.g., butyrylcholinesterase, can bind to the modified BZE–AChE.

The competitive immunoassay allowed the detection of cocaine with a dynamic range from 10^{-9} to 10^{-7} M. The organophosphate chlorpyrifos-oxon could be detected in concentrations from 10^{-6} down to 10^{-8} M after 20 min of injection time (equals to 500 μL sample volume.

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1. Introduction

Each year thousands of tons of organophosphates (OP) are used worldwide (FAOSTAT, 2004). These compounds are highly toxic, because they bind irreversibly to the active site of acetylcholinesterases (AChE). That is why most of the (bio)sensors focus on the AChE's active site and its inhibition (Arduini et al., 2007; Schulze et al., 2003). We developed a binding platform for acetylcholinesterases that takes advantage of a very unique feature of these enzymes, i.e. the peripheral anionic site (PAS). This binding site is located near the gorge that leads to the enzyme's active site. It is known to bind and align the substrate prior to its hydrolysis at the active site (Szegletes et al., 1999). The PAS is also suspected to be involved in amyloid aggregation during Alzheimer's disease (De Ferrari et al., 2001). The therapeutical addressing of this binding site was investigated by Nunes-Tavares et al. (2002). They studied the binding of various tricyclic antidepressants to AChE from the electric eel. Because of its chemical structure the propidium cation can also be classified among these compounds. Its strong interaction with acetylcholinesterase has already been found in 1975 (Taylor and Lappi, 1975). In 2003 the complex between propidium and murine AChE was solved by X-ray crystallography (Bourne et al., 2003) confirming its binding at the peripheral anionic site. Recently, propidium has been employed as a recognition element for acetylcholinesterases on piezoelectric sensors (Teller et al., 2006). It was also found that the deposition of AChE on propidium-modified, piezoelectric sensors does not block its enzymatic activity (Halamek et al., 2006), thus making it possible to investigate the inhibition of the surface-bound enzyme.

The chemical modification of enzymes has a long tradition in biosensorics. Established coupling techniques (Hermannson, 1995) are used to covalently bind biotin, lipids, antigens and proteins to enzymes. The avidin–biotin-system has been extensively studied for the interlinking of biomolecules or for their immobilization onto surfaces and was reviewed elsewhere (Wilchek and Bayer, 1989). The use of lipids as protein anchors is a widely applied concept in nature and has received great attention in the field of sensorics in the last years (Tanaka and Sackmann, 2005). Even the chemical coupling of one enzyme to another was successfully applied. Makower et al. (1997) reported the use of peroxidase as a reporter enzyme

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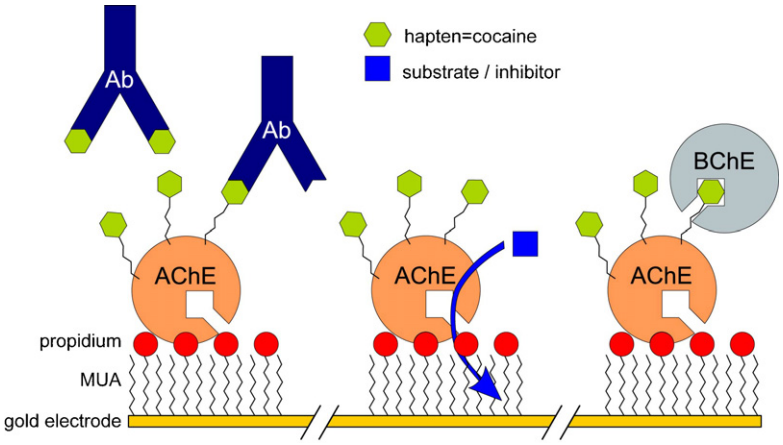

Fig. 1. Binding scheme of BZE–AChE on the propidium-modified QCM sensor. Left: interaction with the anti-BZE-antibody, Middle: substrate conversion by the BZE–AChE or inhibition of the immobilized enzyme, Right: interaction with huBChE. The enzymes are depicted as monomers for clarity.

after coupling to cholinesterase. Last not least the enzyme–antigen conjugates have a long history in the field of ELISA techniques (Tijssen, 1985).

This approach, i.e., the usage of an enzyme–antigen conjugate, has also been used here, but in a different manner. Usually, the antibody is immobilized and a haptenized enzyme, e.g., dinitrofluorobenzene coupled to glucose oxidase (Schuhmann et al., 1991), is used to quantify the amount of free antigen in a competitive assay. Here we used the hapten-enzyme conjugate – benzoylecgonine–acetylcholinesterase (BZE–AChE) – as a binding platform on the piezoelectric crystals for the fraction of anti-BZE-antibody that is not blocked by cocaine (see Fig. 1). This way the mass of the antibody itself serves the measuring signal and no further labeling is needed. Even another enzyme – butyrylcholinesterase (BChE) – can be used to build such modular assembly of recognition sites. Since it lacks a PAS the BChE cannot interact with propidium (Teller et al., 2006). On the other hand BChE is known to be a binder of cocaine (Sun et al., 2002). That is why it should exclusively bind to the haptenized enzyme. The used electrochemical quartz crystal microbalance (EQCM) device allows the simultaneous recording of piezoelectric (mass-dependent) and amperometric signals. This ability was used to determine the activity of the surface-bound BZE–AChE and the inhibition of the former by an organophosphate. The analytes cocaine and chlorpyrifos-oxon were chosen with respect to their analytical relevance (Das, 1993; FAOSTAT, 2004) and to achieve a low interference between the two measured signals.

2. Materials and methods

Enzymes

EeAChE: acetylcholinesterase from *Electrophorus electricus* (EeAChE) was provided from Fluka (Buchs, Switzerland) Lot 44487/1-34802163. The enzyme is a tetramer and has a mass of 240 kDa.

huBChE: human butyrylcholinesterase was kindly provided from Roche (Mannheim, Germany). The enzyme is a tetramer and has a mass of 360 kDa.

Buffers

- MES buffer (0.5 M pH 5.5, 0.1 M pH 6.6), 2-morpholinoethansulfonic acid monohydrate (Sigma–Aldrich, Steinheim, Germany), adjusted with sodium carbonate of the same concentration (Riedel de-Haen, Seelze).
- 50 mM phosphate buffer pH 7.5 according to Sörensen, potassium phosphate monobasic and sodium phosphate dibasic dihydrate (Merck, Darmstadt).
- 40 mM Britton-Robinson-I-buffer pH 8.0, acetic acid (Fluka, Buchs), boric acid (Sigma–Aldrich, Steinheim), phosphoric acid (Merck, Darmstadt).

Chemicals

N-(3-Dimethylaminopropyl)- *N*′-ethyl-carbodiimide · HCl (EDC), dimethylformamide (DMF), *N*-methylmorpholine (NMM), ascorbic acid, acetylthiocholine iodide, 5, 5′-dithiobis(nitrobenzoic acid) (DNTB) and pepsin (from hog stomach) were obtained from Fluka (Buchs, Switzerland).

N-Hydroxysuccinimide (NHS), *N*, *N*′-dicyclohexylcarbodiimide (DCC), acetylcholine chloride, propidium iodide, 11-mercaptoundecanol (MU), 11-mercapto-undecanoic acid (MUA), *N*-bromo-succinimide (NBS) and cocaine hydrochloride were purchased from Sigma (Steinheim, Germany).

Coomassie brilliant blue dye and BSA standard solution were from Pierce, sodium hydroxide from Roth (Karlsruhe, Germany). The Vivaspin centrifuge tube with a cellulosetriacetate membrane and a cut-off of 30 kDa were obtained from Vivascience (Hannover, Germany). The anti-cocaine-antibody (anti-BZE-Ab) was a polyclonal antibody from sheep. This antibody and benzoylecgonine-1,8-diamino-3,4-dioxaoctane (BZE-DADOO, Lot: BMO 15220678 Charge 02) were kindly provided as a gift from Boehringer Mannheim.

Chlorpyrifos-oxon (CPO) was purchased from Dr. Ehrenstorfer GmbH (Augsburg, Germany).

2.1. Measurement of protein concentration

The protein concentration of the cholinesterase samples was measured as described by Bradford (1976). Samples were diluted in 0.9% NaCl. Equal volumes of sample and coomassie brilliant blue were mixed in microtiterplate wells and incubated for 10 min. The absorbance of the resulting complex was measured at 595 nm. A BSA standard solution in the range from 2.5 to 20 $\mu g\,mL^{-1}$ was used for calibration.

2.2. Activity determination of cholinesterases

The enzymatic activity of cholinesterases was measured according to the method of Ellman et al. (1961). The samples containing cholinesterase were diluted in 40 mM Britton-Robinson-I

buffer pH 8. The assay was carried out in a microtiter-plate. Each well contained 210 μL of 40 mM Britton-Robinson-I buffer pH 8, 30 μL 25 mM DTNB and 30 μL of the pre-diluted sample. The reaction was started by adding 30 μL of the substrate acetylthiocholine. The increase in absorbance at 412 nm was measured. The resulting slope was used for the calculation of cholinesterase activity using the molar extinction coefficient $\varepsilon = 13,300\,\mathrm{L\,mol^{-1}\,cm^{-1}}$. 1 unit corresponds to the amount of enzyme which hydrolyzes 1 μmol substrate per minute.

2.3. Chemical modification of piezoelectric sensors

The piezoelectric sensors were purchased from ICM (Oklahoma, USA). The quartz plates with a basic resonance frequency of 10 MHz were coated with gold electrodes and optically polished by the manufacturer. At first the sensors were washed for 2 h in acetone and dried with nitrogen. The cleaned sensors were incubated in a mixture of three volume parts 5 mM MU and one volume part 5 mM MUA in ethanol for 48 h. In this time a mixed monolayer of MU/MUA self-assembles on the gold surface. A 1 mM stock solution of propidium in DMF and a 5 mM stock solution of DCC in DMF were prepared. The final reaction mixture was comprised of 5 volume parts of the propidium stock solution, 1 volume part of the DCC stock solution and 14 volume parts of DMF. The sensors with the self-assembled MUA/MU layer were immersed in 800 μL of this reaction mixture and incubated over night. Finally, the sensors were successively washed with DMF, 96% ethanol and deionized water.

2.4. Chemical modification of the acetylcholinesterase

For the chemical modification of EeAChE the approved EDC/NHS coupling technique was used. Cholinesterase, EDC and NHS were mixed according to a molar ratio of 1:1000:2000. In this case 2.08 mg of EeAChE (≙8.67 nmol) were dissolved in 500 μL of 0.5 M MES buffer pH 5.5. To this protein solution 50 μL of a 181 mM solution of EDC in MES buffer and 25 μL of a 683 mM solution of NHS in MES buffer were added. This mixture was incubated in an overhead shaker at room temperature for 2 h. The next step differed for the two obtained BZE–AChE conjugates. The reaction took place without and in presence of an excess of cocaine, yielding conjugate A and B, respectively.

Conjugate A: BZE-DADOO (3.89 mg ≙9.27 μmol) was dissolved in 325 μL of 0.1 M MES buffer pH 6.6 and added to the reaction mixture. This provides a 1000-fold molar excess of BZE-DADOO to the cholinesterase. A total of 60 μL of NaOH (1 M stock solution) were added to raise the resulting pH of the reaction mix above 6.0 (tested with pH test stripes by Machery & Nagel). The resulting solution was incubated in the overhead shaker at room temperature for 4 h.

Conjugate B: 3.87 mg BZE-DADOO (≙9.22 μmol) was dissolved in 100 μL of 0.1 M MES buffer pH 6.6. Furthermore, 31.48 mg cocaine (≙92.64 μmol) was dissolved in 300 μL of the same buffer. The 100 μL of the BZE-DADOO solution and 275 μL of the cocaine solution were added to the NHS-activated AChE. 70 μL of 1 M NaOH was added in drops to increase the pH of the reaction mixture above 6.0 (tested with pH test stripes by Machery & Nagel). The final concentration of cocaine in the reaction mixture was 100 mM. The resulting solution was incubated as described above in the overhead shaker at room temperature for 4 h.

After the reaction both conjugates were filtrated in 2 mL Vivaspin concentrators at 3000 × *g* and 4 °C for 10 min. After each run the filtrate was discarded and 1 mL of 50 mM phosphate buffer pH 7.5 was added to the concentrate. From the third run a mixture of 100 μL 30 mM acetylcholine and 900 μL buffer was added instead of pure buffer. This was done to remove non-coupled BZE-DADOO and excess cocaine, that could potentially bind to the cholinesterase's active site. After another three runs pure buffer was used to wash out excess acetylcholine and its hydrolysis product. Phosphate buffer was added to the final concentrate to give a volume of 1 mL. The protein concentration and activity of the modified AChE were determined using Bradford's and Ellman's method, respectively (see above).

2.5. Piezoelectric measurements

The piezoelectric detector was obtained from J. Kitlička (Brno, Czech Republic). The corresponding measuring software has been developed by Prof. P. Skládal from the Masaryk University (Brno, Czech Republic). The theoretical resolution of this device is 1 mHz and data acquisition rate is 1 point per second. The set-up also includes a peristaltic pump from Abimed Gilson (Langenfeld, Germany) and a custom-made flow-through cell, which hold the quartz crystal sensor. The flow cell has an internal volume of ca. 8 μL when a QCM sensor is mounted. The components are arranged in such a way that the pump is drawing liquid from the measuring cell. By doing this, fluid pulsations are minimized and a lower noise of the frequency baseline can be obtained. The flow rate was adjusted to 20 $\mu\mathrm{L\,min^{-1}}$. For all experiments 50 mM phosphate buffer pH 7.5 (Sörensen buffer) was used. Buffer was allowed to flow for 30 min to stabilize the baseline before using a fresh sensor. A measuring cycle usually consisted of 3 min baseline recording, 5 min sample injection, 8 min buffer flow and 3 min regeneration. The regeneration agent was 2 $\mathrm{mg\,mL^{-1}}$ pepsin in 50 mM phosphate buffer adjusted to pH 2.

2.5.1. Binding of anti-BZE-antibody to BZE–AChE

The following measuring scheme was used to evaluate the binding of the antibody to the BZE–AChE conjugate:

- 3 min flow of buffer;
- 5 min injection of 50 $\mu\mathrm{g\,mL^{-1}}$ BZE–AChE;
- 5 min buffer flow;
- 5 min injection of different concentrations of anti-BZE-antibody;
- 5 min flushing with buffer;
- 5 min regeneration with pepsin solution pH 2.

The antibody stock solution was diluted with phosphate buffer to give 2, 5, 10, 25 and 50 $\mu\mathrm{g\,mL^{-1}}$. The resulting binding curves were non-linearly fitted to a one-to-one binding model as described by Teller et al. (2006) and Zeravik et al. (2006) using Eq. (1) to yield the rate and equilibrium constants.

$$\Delta f(t) = \frac{k_\mathrm{a} \cdot c \cdot \Delta f_\mathrm{max}}{k_\mathrm{a} \cdot c + k_\mathrm{d}} \cdot (1 - \mathrm{e}^{(k_\mathrm{a} \cdot c + k_\mathrm{d})(t_0 - t)}) \quad (1)$$

The two rate constants k_a and k_d are obtained from the measured frequency shift $\Delta f(t)$. The molar concentration of the antibody is denoted by *c*. Δf_max represents the frequency difference obtained by complete saturation of the sensor surface. The additional parameter t_0 accounts for the injection delay.

2.5.2. Competitive assay for cocaine

For the competition experiments with cocaine and antibody the same measuring scheme as for the kinetic evaluation was used but with the addition, that the antibody was pre-incubated with cocaine in varying concentrations. The antibody concentration was fixed at 25 $\mu\mathrm{g\,mL^{-1}}$ for these experiments.

A 10 mM stock solution of cocaine in 50 mM phosphate buffer pH 7.5 was prepared and diluted in a sequence of 10-fold dilutions

using the same buffer. Further intermediate dilutions were added in the dynamic range of the response curve. 30 min before injection 30 μL of any cocaine dilution were mixed with 270 μL of the antibody solution to give a final cocaine concentration between 10^{-6} and 10^{-9} mol L^{-1}.

Since the injection time is too short to reach the binding equilibrium, the slope of the resulting frequency change was calculated and used for further evaluations. To minimize inter-day fluctuations all slopes were normalized to the one obtained from the injection of antibody without hapten (270 μL + 30 μL of phosphate buffer). A total of three concentration series was assayed.

2.5.3. Binding of huBChE to BZE–AChE

In this case the injection scheme was slightly modified to evaluate the interaction between butyrylcholinesterase and the BZE–AChE conjugate:

- 3 min flow of buffer;
- 5 min injection of 50 μg mL^{-1} BZE–AChE;
- 5 min buffer flow;
- 5 min injection of 100 μg mL^{-1} huBChE; free enzyme or preincubated with cocaine;
- 5 min flushing with buffer;
- 5 min regeneration with pepsin solution pH 2.

2.6. Amperometric measurements

The piezoelectric device was also used for amperometric measurements. The gold electrode on the quartz plate that is facing the liquid is used as the working electrode in this mode. Another custom-built flow cell was employed for the combined piezoelectric/amperometric experiments. This cell holds a Ag/AgCl reference electrode from Microelectrodes, Inc. (Bedford, USA) and a platinum auxiliary electrode. The internal volume of this cell is comparable to the one used exclusively for piezoelectric experiments. The activity of the in situ immobilized AChE was monitored via the enzymatic hydrolysis of acetylthiocholine (ATC) to acetate and thiocholine. The thiocholine was then oxidized at the working electrode using a potential of +200 mV vs. Ag/AgCl. Dilutions of the ATC substrate in buffer at pH 7.5 show a significant self-hydrolysis throughout a series of measurements. That is why we used a stock solution of 30 mM prepared in deionized water. This stock solution was kept on ice and freshly diluted to the final working concentration of 0.5 mM with running buffer just before the injection into the flow system. Also the working potential was only applied during injection of ATC and subsequent buffer flow. At all other times of the experiment the working electrode was disconnected.

Chlorpyrifos-oxon (CPO) was obtained as a solution in cyclohexane (1 ng/mL). Aliquots of the stock solution were dried in an rotation evaporator at 20 mbar and room temperature. The remaining solid was re-dissolved in phosphate buffer.

The injection scheme was as follows:

- 3 min flow of buffer;
- 5 min injection of 50 μg mL^{-1} BZE–AChE;
- 3 min buffer;
- 3 min injection of 0.5 mM acetylthiocholine (ATC), freshly prepared solution;
- 3 min buffer flow;
- 6 min repeated injection of ATC and buffer;
- 3 min injection of organophosphate;
- 3 min flushing with buffer;
- 12 min: two injections of freshly prepared ATC as described above;
- 5 min flow of pepsin solution pH 2.

Table 1
Comparison of the two BZE–AChE conjugates; the relative binding is the normalized frequency change obtained after 5 min injection of 50 μg mL^{-1} BZE–AChE

	Conjugate A	Conjugate B
Protein concentration in μg mL^{-1}	945	1335
Specific activity (% orig.)	2	66
Relative binding in %	100	98.5

3. Results and discussion

3.1. Properties of the modified acetylcholinesterases

Two Conjugates between BZE-DADOO and EeAChE were obtained: one in the absence of cocaine (conjugate A) and one in the presence of cocaine (conjugate B). Table 1 lists the properties of the two obtained BZE–AChE conjugates. In both cases almost all of the protein can be recovered after the coupling and purification process. There is but a huge difference in the specific activity of the resulting modified acetylcholinesterase. Conjugate A which has been synthesized in the absence of cocaine shows only 2% of the original specific enzyme activity. In case of conjugate B an excess of cocaine during the coupling process results in preserving two thirds of the original activity. This can be explained with the fact that cocaine is a weak inhibitor of cholinesterases (Xie et al., 1999). Therefore, is it possible that the BZE-DADOO – a cocaine derivative – binds close or directly at the active site. If there are any NHS-activated residues nearby those binding sites, it is imaginable that BZE-DADOO will be fixed at this position by chemical coupling. The resulting enzyme subunit will then show little to no activity like, for example, in the case of conjugate A. This inactivation can be successfully circumvented by the addition of an excess of free cocaine to the reaction mixture as the synthesis of conjugate B shows. In this case cocaine will displace BZE-DADOO at the active site and thus prevent the inactivation by chemical coupling. When injected into the flow system in a concentration of 50 μg mL^{-1}both conjugates show a similar binding to the propidium sensor, i.e., the frequency change after 5 min. This indicates that the propidium binding site in contrast to the active site is not affected by the chosen coupling method.

3.2. Binding of anti-BZE-antibody to BZE–AChE

3.2.1. Kinetic evaluation of the antibody binding

The kinetic rate constants were obtained directly from the non-linear fit of the binding records to the integrated rate equation (Eq. (1)). The association rate constant k_a was determined as 12911 ± 1147 L mol^{-1} s^{-1}. The corresponding dissociation rate constant k_d was found to be $2.89 \times 10^{-3} \pm 1.49 \times 10^{-3}$ s^{-1}. This leads to equilibrium constant K_D of 2.239×10^{-7} mol L^{-1}. While the k_a value is comparable to the number obtained in earlier investigations of the anti-BZE-antibodies ($k_a = 22,200$ L mol^{-1} s^{-1}), the dissociation rate constant k_d differs by several orders of magnitude (9.60×10^{-5} s^{-1}Halamek et al., 2002). This is mainly due to a difference in the used evaluation method. Halamek et al. used a two step method to obtain the binding rate constants, i.e., a non-linear fit to obtain pseudo-rate constants k_{obs} and a linear regression of the k_{obs} to yield the desired k_a and k_d. Here we used total non-linear approach to derive the rate constants directly (see Eq. (1)). Also the authors of the original paper consider their k_d value to be "not precise enough as k_d was quite close to zero". Still a higher apparent dissociation rate can be expected, if the dissociation of the (non-

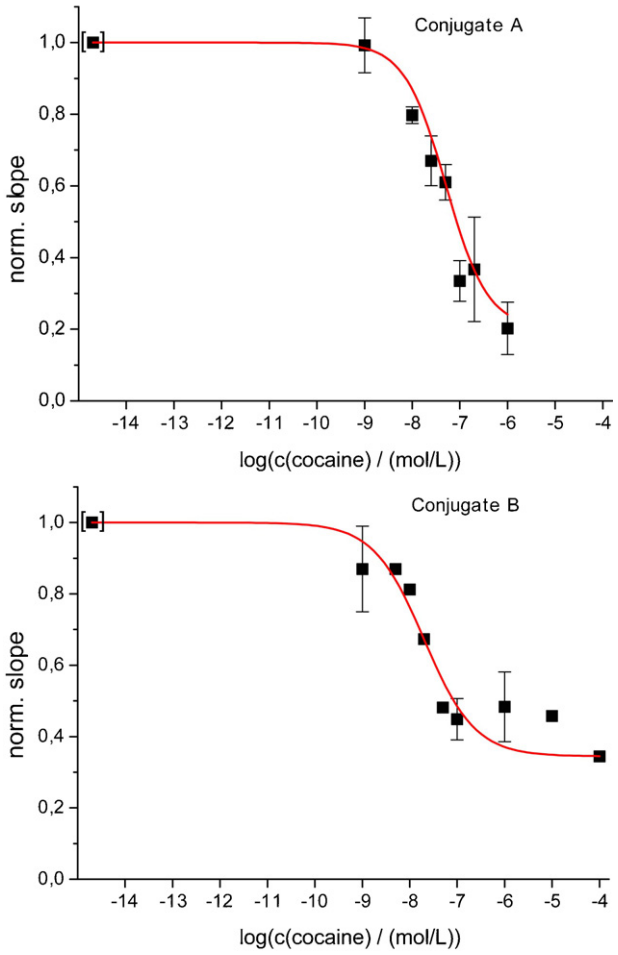

Fig. 2. Competitive assay for cocaine using BZE–AChE conjugate A (left) and B (right) as a recognition layer; the data points in brackets represent the signal of antibody without cocaine.

covalently) bound BZE–AChE conjugate is taken into account.[1] This fact was omitted when the fitting routine was chosen, as the resulting model would have been more complex.

3.2.2. Competitive assay with cocaine and the anti-BZE-antibody

Two assays were examined to compare the BZE–AChE conjugates A and B. The normalized slope of antibody binding was plotted vs. the cocaine concentration used. Fig. 2 shows the results. In both cases an excess of cocaine can prevent the antibody from binding to the BZE–AChE layer. There is but a difference in the remaining non-specific binding at high concentrations of cocaine. On the other hand both conjugates show a similar test mid-point: $c_{50\%} = 4.40 \times 10^{-8}$ mol L^{-1} for conjugate A and $c_{50\%} = 1.77 \times 10^{-8}$ mol L^{-1} for conjugate B, respectively. These results are comparable to other reported assays ($c_{50\%} \approx 1 \times 10^{-8}$ mol L^{-1} Halamek et al., 2002), although a limit of detection (LOD) as low as the reported 10^{-10} mol L^{-1} could not be reached with our approach. The main difference between our system and the one described by Halamek et al. (2002) is the much lower surface density of antigen. Here we use

[1] In this case the observed k_d can be represented as the sum of two k_d-values: one for the interaction between propidium and BZE–AChE and one for the binding of the anti-BZE-antibody to BZE–AChE. Indeed the calculated k_d mentioned above is higher than the one published for the interaction of propidium and electric eel AChE (see Teller et al., 2006).

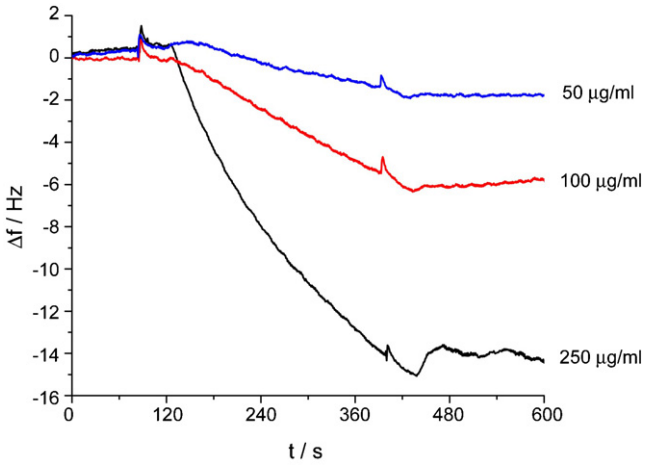

Fig. 3. Interaction between huBChE and the BZE–ACHE layer; injection of different concentrations of huBChE after injection of 50 µg mL^{-1} BZE–AChE.

a freshly deposited layer of haptenized acetylcholinesterase as compared to BZE-DADOO being coupled directly to MUA and assembled as a monolayer. A similarly low LOD (0.38×10^{-9} mol L^{-1}) was reported by Bauer et al. (1998) where BZE-DADOO was coupled onto a chromatographic affinity support. The advantage of our system is that the depletion of ligands by repeated regeneration and washing can be circumvented by the fresh deposition of BZE–AChE. The underlying propidium sensor has already proved its high potential for re-usability (Teller et al., 2006; Halamek et al., 2006). One sensor could be used to measure the calibration curves for both conjugates (see Fig. 2) with pepsin at pH 2 as the regeneration solution.

3.3. Binding of butyrylcholinesterase to BZE–AChE

The natural isomer (−)-cocaine is a substrate of the human butyrylcholinesterase. HuBChE has been used in sensors with cocaine as the recognition element because of its rather low catalytic conversion rate ($k_{cat} = 3.9\,\text{min}^{-1}$) and moderate affinity ($K_M = 14\,\mu\text{mol L}^{-1}$) for cocaine (Xie et al., 1999; Halamek et al., 2005). As Fig. 3 shows butyrylcholinesterase also binds to the BZE–AChE layer, but the resulting frequency change is lower than the one obtained from antibody binding. This is mainly due to the huge difference in affinity toward cocaine. It is possible to calculate the amount of deposited molecules using the Sauerbrey-equation (Eq. (2), after Sauerbrey, 1959). For a 10 MHz crystal and an electrode diameter of 5.1 mm the sensitivity S equals to 1.16 Hz/ng (see Eq. (3)).

$$\Delta f = \frac{-2{f_0}^2}{\sqrt{\mu_q \rho_q} A} \Delta m \quad (2)$$

$$S = \frac{|\Delta f|}{\Delta m} = 1.16\,\text{Hz/ng} \quad \text{resp.} \quad 0.86\,\text{ng/Hz} \quad (3)$$

Depending on the molecular weight of the binding protein this leads to a corresponding ratio of 3.6 fmol/Hz for BZE–AChE, 2.4 fmol/Hz for huBChE and 5.7 fmol/Hz for the anti-BZE-antibody, respectively. For a consecutive injection of 50 µg mL^{-1} BZE–AChE and 100 µg mL^{-1} huBChE a binding ratio of one BChE-tetramer per five molecules of immobilized BZE–AChE is obtained. On the other hand a binding ratio of 1, 2 and 3 antibodies per BZE–ACHE-tetramer can be reached after injection of 10, 25 or 50 µg mL^{-1} anti-BZE-antibody, respectively. This is another indication for the lower affinity of the BChE in comparison to the anti-BZE-antibody. Nonetheless, the interaction between huBChE and BZE–AChE can be blocked by an excess of cocaine in solution after 30 min of pre-

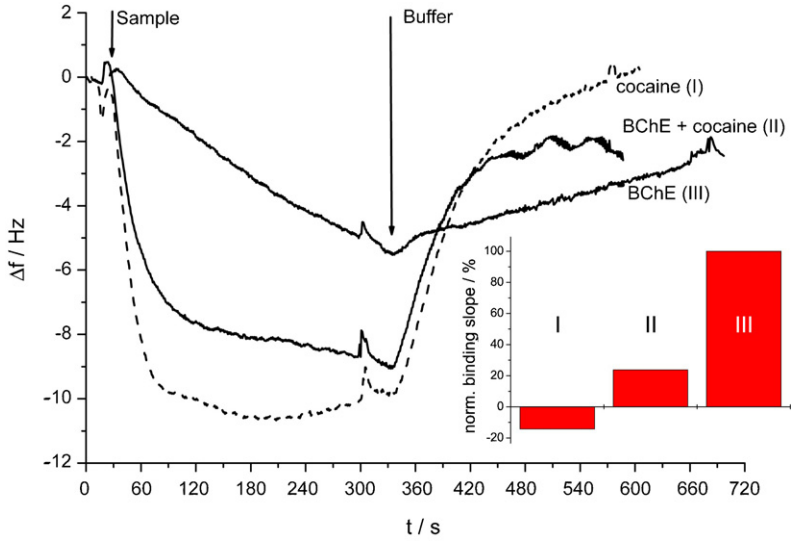

Fig. 4. Blocking the binding huBChE to the BZE–ACHE layer by cocaine; injection of 100 μg mL^{-1} huBChE without and in the presence of 1 mmol L^{-1} cocaine (solid lines); sensor response to 1 mmol L^{-1} cocaine (dashed line); Inset: comparison of the binding slope from 120 to 300 s, normalized to the one obtained by free huBChE.

incubation time (see Fig. 4). The observed fast drop in frequency at the beginning of the injection of huBChE in 1 mmol L^{-1} cocaine can be explained as a reaction of the piezoelectric sensor to the change of viscosity of the relatively highly concentrated solution. When only 1 mmol L^{-1} cocaine is injected into the flow system a similarly fast frequency change is recorded. The part from 120 to 300 s makes the main difference of the three curves. The slope for the interaction of free huBChE is much steeper as in case of the huBChE in presence of 1 mmol L^{-1} cocaine. For the sample that contains only cocaine there is hardly any change of frequency in this time window. Although this confirms the specificity of the binding of huBChE to BZE–AChE, the low affinity of the butyrylcholinesterase toward cocaine complicates the use of this system for analytical purposes.

3.4. Inhibition of BZE–AChE with chlorpyrifos-oxon

As described above the activity of the adsorbed BZE–AChE layer was measured by the hydrolysis of acetylthiocholine and the subsequent oxidation of the product at the working electrode. Fig. 5 shows a sample record of the obtained amperometric data. After the deposition of BZE–AChE on the propidium sensor (A) its initial activity was determined by injection of the ATC substrate (B). The injection of the inhibitor chlorpyrifos-oxon (C) is followed by the measurement of the remaining activity (D). The experiment is concluded by the sensor regeneration (E). The relative peak height of the current measured after CPO inhibition is used as an indicator for the remaining active fraction of immobilized AChE. Fig. 6 shows the dependence of the remaining AChE activity on the applied CPO concentration. The dynamic range of this assay is between 5×10^{-6} down to 5×10^{-8} M CPO. These results are comparable to the work of Jeanty et al. (2002). They published a flow-injection system with EeAChE immobilized in a photo-crosslinkable polymer. In this case they could detect an inhibition by CPO in a concentration range from 1.3×10^{-6} down to 6.3×10^{-8} M. Still, these values are rather high as compared to other AChE-based sensors (Arduini et al., 2007; Sotiropoulou et al., 2005). The first paper utilizes highly toxic nerve agents, such as sarin and VX, to reach fast inhibition at low concentrations. On the other hand, a complicated set-up and high safety precautions are necessary for the sensor calibration. The paper by

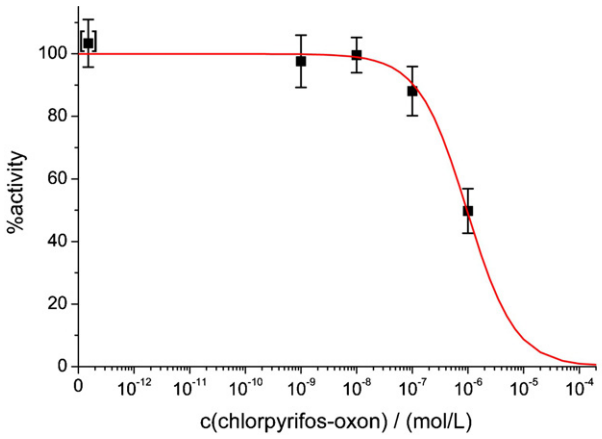

Fig. 6. Concentration dependence for chlorpyrifos-oxon; the data point in brackets represents the relative activity after 20 min buffer flow instead of chlorpyrifos-oxon.

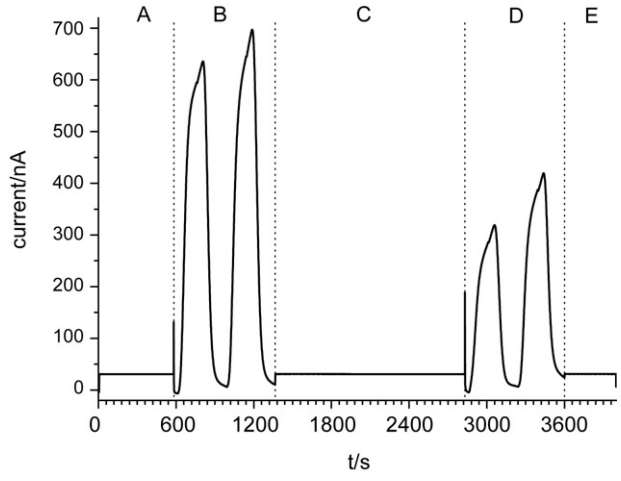

Fig. 5. Sample record for the inhibition of surface-bound BZE–AChE; (A) injection of BZE–AChE, (B) initial activity measurement, (C) injection of chlorpyrifos-oxon (here 1×10^{-6} M), (D) final activity determination, (E) regeneration.

Sotiropoulou et al. describes the use of genetically engineered AChE from *Drosophila melanogaster*. The enzyme was immobilized at a porous carbon electrode. In this case the authors claim a detection limit of 10^{-17} M for dichlorvos and linear range over not less than 6 orders of magnitude which makes these results somewhat questionable.

4. Conclusions

Here we describe a combined piezoelectric/amperometric sensor that is based on the modular assembly of different recognition elements. The basis of the system is the well-described propidium-modified QCM sensor (Teller et al., 2006; Halamek et al., 2006). Acetylcholinesterase was chemically modified by BZE-DADOO, thus providing an additional recognition element for anti-cocaine-antibodies or butyrylcholinesterase, respectively. Using the polyclonal antibody it was possible to determine cocaine in dynamic range of 10^{-7} to 10^{-9} mol L^{-1}. Furthermore, the specific interaction of huBChE and the BZE–AChE layer was proved. At the same time the in situ inhibition of adsorbed BZE–AChE by the organophosphate chlorpyrifos-oxon could be monitored by amperometric activity measurements. The use of inhibitors which have a much higher inhibition constant could provide similar LOD values as recently described for VX and sarin (Arduini et al., 2007).

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