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Piezoelectric affinity sensors for cocaine and cholinesterase inhibitors

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Abstract

We report here the development of piezoelectric affinity sensors for cocaine and cholinesterase inhibitors based on the formation of affinity complexes between an immobilized cocaine derivative and an anti-cocaine antibody or cholinesterase. For both binding reactions benzoylecgonine-1,8-diamino-3,4-dioxaoctane (BZE-DADOO) was immobilized on the surface of the sensor. For immobilization, pre-conjugated BZE-DADOO with 11-mercaptomonoundecanoic acid (MUA) via 2-(5-norbornen-2,3-dicarboximide)-1,1,3,3-tetramethyluronium-tetrafluoroborate (TNTU) allowed the formation of a chemisorbed monolayer on the piezosensor surface.

The detection of cocaine was based on a competitive assay. The change of frequency measured after 300 s of the binding reaction was used as the signal. The maximum binding of the antibody resulted in a frequency decrease of 35 Hz (with an imprecision 3%, $n = 3$) while the presence of 100 pmol l^{-1} cocaine decreased the binding by 11%. The limit of detection was consequently below 100 pmol l^{-1} for cocaine. The total time of one analysis was 15 min.

This BZE-DADOO-modified sensor was adapted for the detection of organophosphates. BZE-DADOO – a competitive inhibitor – served as binding element for cholinesterase in a competitive assay.
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1. Introduction

The detection of toxic compounds including drugs and pesticides is an important but difficult analytical task. Separation techniques such as high-performance liquid chromatography (HPLC) and gas chromatography/mass spectrometry (GC/MS) have been applied, but they require sophisticated instrumentation. Antibody-based techniques are inherently less complicated and thus appropriate in forensic and environmental analysis. A fast and sensitive method for monitoring toxins is the non-competitive immunoenzymetric assay (IEMA) [1]. The high sensitivity of this method is obtained by using an enzyme label, which requires the synthesis of labeled immunoconjugates. In a previous paper [2], we described a piezoelectric immunosensor for the determination of cocaine, which is based on the measurement of mass changes resulting from the formation of affinity complexes thus avoiding any need for labeling.

Today cocaine is one of the most commonly used drugs. If overdosed, it acts as an anti-muscarinic drug inhibiting gastric motility and increasing the likelihood of gastric ulceration and perforation [3]. There is evidence that butyrylcholinesterase (BChE, EC 3.1.1.8) in blood is the major detoxicating system of cocaine to give ecgonine methylester and benzoic acid [4]. The rate of cocaine hydrolysis by AChE – as expressed by k_{cat} of 0.0651 s^{-1} – is almost neglectable as compared with the diffusion controlled splitting of acetylcholine (k_{cat} = 25,000 s^{-1}). Therefore, the enzyme–cocaine complex is split only very slowly and, consequently, cocaine acts as a competitive inhibitor of the hydrolysis of the "natural" substrates.

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Organophosphorous compounds are nowadays widely used pesticides. Pesticide determination utilizing cholinesterase as the target molecule is usually based on monitoring the decreased enzyme activity caused by the action of the inhibitor. To avoid complication with the irreversible inhibition, the enzymatic activity of cholinesterase was not addressed in this paper; the binding of ChE to its reversible inhibitor (benzoylecgonine-1,8-diamino-3,4-dioxaoctane (BZE-DADOO)) was measured instead. This process was monitored with a mass sensitive piezoelectric quartz crystal (quartz crystal nanobalance (QCN)).

Several piezoelectric sensors for the detection of low molecular compounds like pesticides [5–7], toxins [8] or drugs [2] have been reported in recent years. A piezoelectric biosensor with reversibly coupled (via His-tag) paraoxon for the binding of tetrameric butyrylcholinesterase to the active site of the enzyme has also been described by us [6]. The commercially available devices have recently been reviewed [5].

This paper presents the development of a piezoelectric affinity sensor for the detection of cocaine by antibodies and of organophosphates by acetylcholinesterase, respectively. In both cases, the assay is performed in a competitive mode indicating the binding of the free antibody or the non-inhibited AChE to the immobilized BZE-DADOO by the piezoelectric nanobalance.

2. Experimental

2.1. Reagents

N-methyl-morpholine (NMM) was from Fluka (Buchs, Switzerland) and dimethylformamide (DMF) from Mallinckrodt Baker (Griesheim, Germany). 2-(5-Norbornen-2,3-dicarboximido)-1,1,3,3-tetramethyluronium-tetrafluorborat (TNTU) was from Calbiochem (San Diego, USA), cocaine hydrochloride and 11-mercaptoundecanoic acid (MUA) were supplied by Sigma (Deisenhofen, Germany), bovine serum albumin, affinity purified antibodies against BZE-DADOO as a sheep polyclonal antibody IgG were kindly gifted by Boehringer (Mannheim, Germany) and were used as received. Benzoylecgonine-1,8-diamino-3,4-dioxaoctane was from Biosyntan (Berlin, Germany). Acetylcholinesterase (specific activity 1100 U mg^{-1}) from *Electrophorus electricus* (EeAChE) was obtained from Fluka (Buchs, Switzerland). This enzyme is a tetramer with a molecular weight of 240 kDa. Diisopropylfluorophosphate was from Serva (Heidelberg, Germany). Micro-dialysis tubes Centrisart I (cut-off MW 20 kDa) were from Sartorius (Göttingen, Germany).

2.2. Sensors

Sensors were purchased from Elchema (Potsdam, NY, USA). The quartz discs (10 MHz, AT-cut, 14 mm in diameter) were coated on both sides with gold electrodes (5 mm diameter).

2.2.1. Immobilization of ligands

At the beginning, the metal electrodes of the sensor were always carefully washed for 2 h in acetone followed by distilled water and dried in the air. MUA (1.77 mg, 8.0 μmol) and TNTU (2.9 mg, 7.9 μmol) were dissolved in a mixture of 1 μl NMM (9.6 μmol) and 100 μl DMF and incubated for 15 min at room temperature. BZE-DADOO (200 μl, 10 mmol l^{-1} solution) was dissolved in 0.1 M carbonate buffer pH 8.5. Twenty-two microliters of MUA–TNTU solution and 200 μl of BZE-DADOO solution were mixed together and incubated for 90 min at room temperature. The MUA–BZE-DADOO conjugate thus obtained was stored in a freezer. The sensing layer was obtained by formation of the MUA–BZE-DADOO conjugate monolayer on the electrode surface by the incubation of 30 μl of the conjugate for 48 h at 4 °C in a wet chamber. After washing with distilled water and drying, the sensor was stored in the refrigerator.

2.3. Experimental flow-through system

The modified crystal was fixed in a thin layer flow through cell between two soft rubber o-rings. Only one side of the crystal was in contact with the flowing solution. The experiments were performed at room temperature, 50 mmol l^{-1} phosphate buffer pH 7.0 (pH 7.5 was chosen for ChE experiments) were used as a working solution continuously flowing through the cell. Deionized water was used for all experiments. The output tube from the cell was connected to the peristaltic pump Minipuls 3 (Gilson, France). A flow rate of 20 μl min^{-1} was used for the experiments. The Abimed® tubes (Ø 0.2 mm) were used for connections. The electrodes of the crystal were connected to a gate oscillating circuit based on the integrated oscillator driver 74LS320. The output frequency was measured using the frequency counter UZ2400 (Grundig, Brno, Czech Republic) controlled by a computer. In the experiments with cholinesterase we used the MultiLab 3900 piezoelectric instrument (Kitlička, Brno, Czech Republic, theoretical resolution 1 mHz) controlled by a computer. For both instruments, the software LabTools ver. 1.1 (P. Skládal, Brno, Czech Republic) was used for measurements, data storage (1 s per one point) and evaluation.

2.4. Measuring procedure

2.4.1. Immunosensor for cocaine detection

Before the first use of a fresh side of the modified crystal, the carrier buffer was allowed to flow through the cell for 15 min in order to stabilize the frequency level. A flow (3 min) of BSA solution (1 mg ml^{-1}) followed to saturate any non-specific binding sites. One measuring cycle consisted of the following steps:

- 1 min flow of the phosphate buffer to achieve a stable initial frequency;
- 3–10 min flow of the buffer solution containing antibody (in selected experiments a mixture of antibody and cocaine was used); recording of the binding curve;
- 5 min flow of the buffer to determine the resulting final change of frequency;
- 4 min flow of 500 mmol l^{-1} formic acid to dissociate the ligand–antibody complex and regenerate the sensing surface of the crystal.

The delay between the change of the solution at the input tube and its entering of the measuring cell was 60 s. The determination of free cocaine was performed competitively. In a plastic microtube, the selected amount (14 μg ml^{-1}) antibody was mixed and stirred shortly with cocaine in the presence of phosphate buffer. The concentration of antibody was chosen to achieve the appropriate response of the piezosensor, i.e. here it represented a signal of approximately 35 Hz. The mixture was allowed to incubate for a selected time interval, then it was introduced to the flow system with the crystal and the binding curve was recorded.

2.4.2. Sensor for organophosphate detection

Before the first use of a fresh side of the modified crystal, the carrier buffer was allowed to flow through the cell for 15 min in order to stabilize the frequency. One measuring cycle consisted of the following steps:

- 3 min flow of the phosphate buffer to achieve a stable initial frequency;
- 5 min flow of the buffer solution containing cholinesterase; recording of the binding curve;
- 5 min flow of buffer to determine the resulting change of frequency;
- 5 min flow of the regeneration solution;
- 5 min flow of the phosphate buffer to achieve a stable initial frequency.

For regeneration, 1 mol l^{-1} formic acid solution was used to dissociate the BZE-DADOO–cholinesterase complex. The sensor was used for more than 40 regeneration steps.

2.5. Kinetic evaluation of experimental data

The binding of the free partner B to the immobilized partner A and the dissociation of the resulting complex (AB) is often assumed to follow pseudo-first order kinetics and can be represented by Eq. (1):

$$A + B \rightleftarrows AB \tag{1}$$

This reaction can be described by the rate constants of association (k_a) and dissociation (k_d), respectively. The overall rate of complex formation is as follows:

$$\frac{d[AB]}{dt} = k_a[A][B] - k_d[AB] \tag{2}$$

The decrease of frequency f is directly proportional to the attached mass. Using f_m – the frequency change after a complete saturation of the sensor surface with compound B – the concentration of the free affinity partner B is proportional to ($f_m - f$). The rate of the complex AB formation can be expressed as:

$$\frac{df}{dt} = k_a[B](f_m - f) - k_d f \tag{3}$$

where [B] is the concentration of free compound B, which is held constant in a continuously flowing solution. From values of these kinetic parameters, it is possible to determine the equilibrium constant K_a:

$$K_a = \frac{k_a}{k_d} \tag{4}$$

The dependence of the frequency change Δf on time t during affinity interaction between immobilized partner A and free affinity partner B (the binding curve) can be fitted to the equation obtained by integration of Eq. (3):

$$\Delta f = \frac{k_a c f_m}{k_a c + k_d}\{1 - \exp[-(k_a c + k_d)t]\} = f_{eq}[1 - \exp(-k_{obs}t)] \tag{5}$$

To obtain one set of kinetic parameters, binding curves with at least five different concentrations of the free binding partner B are recorded. For each individual curve, calculation of k_{obs} and f_{eq} is performed using the non-linear curve fitting procedure. Consequently, kinetic parameters k_a and k_d can be obtained from the dependence of k_{obs} on c as slope and intercept, respectively.

If the value of k_d is low, it is better to use the dissociation phase of curves (the phase when the compound B is not present in the flowing buffer). Here the calculation of k_d values was performed using the non-linear curve fitting procedure.

3. Results and discussion

3.1. Monitoring of affinity interaction by piezoelectric sensor

Typical experimental signal traces obtained during the binding of anti-cocaine pAb and EeAChE, respectively, to the immobilized BZE-DADOO are recorded in Figs. 1 and 2 together with the regeneration phase.

3.2. Determination of kinetic parameters by piezoelectric sensors

In addition to concentration measurements, piezoelectric sensors are also suitable tools for a kinetic characterization of affinity binding interactions [10]. Samples containing different concentrations of the antibodies or cholinesterase, respectively, were allowed to interact with BZE-DADOO-modified

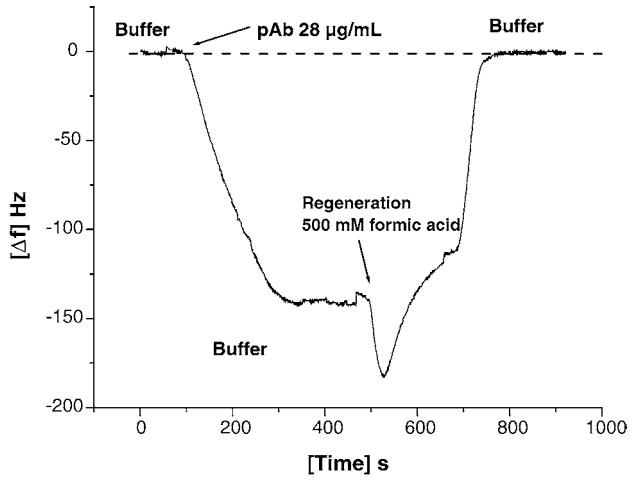

Fig. 1. Experimental trace (change of frequency vs. time plot) representing the binding of the specific pAb to the immobilized BZE-DADOO. The dashed line shows the background signal of the sensor.

sensors placed in a flow-through cell. The association phase was recorded and experimental traces of the interaction of polyclonal antibody, or EeAChE with BZE-DADOO thus obtained are shown in Figs. 3 and 4, respectively.

For each individual trace, the values of k_{obs} and f_{eq} were determined using non-linear curve fitting of the frequency f versus time t traces according to Eq. (5). Plot of k_{obs} values against corresponding concentrations c of antibody or AChE, respectively, leads to a linear dependence. The k_{obs} versus c plot and the values obtained for pAb and AChE binding to the BZE-DADOO sensor are shown in Figs. 5 and 6, respectively.

The obtained values of association and dissociation rate constants for both partners are summarized in Table 1.

Equilibria constants for the binding partners were determined according to Eq. (4) (Table 2).

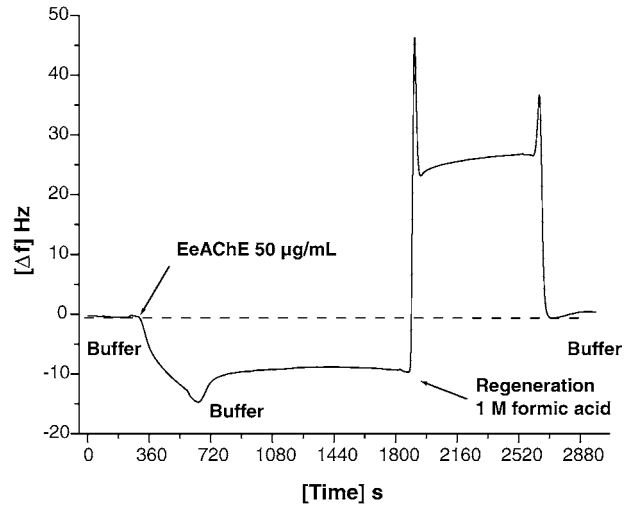

Fig. 2. Typical binding trace of EeAChE interaction with the piezoelectric crystal modified with the BZE-DADOO ligand. The dashed line shows the background signal of the sensor.

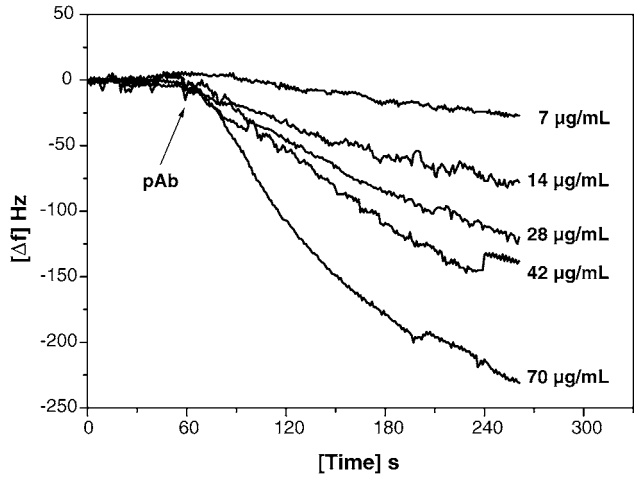

Fig. 3. Binding curves for different concentrations (indicated close to traces) of the polyclonal anti-cocaine antibody to the cocaine derivative BZE-DADOO immobilized on a piezoelectric sensor.

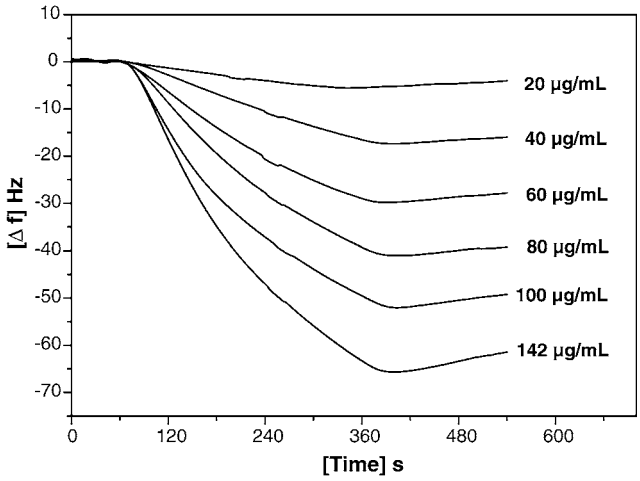

Fig. 4. Concentration dependence (indicated close to traces) of *Electrophorus electricus* acetylcholinesterase binding to the immobilized cocaine derivative BZE-DADOO.

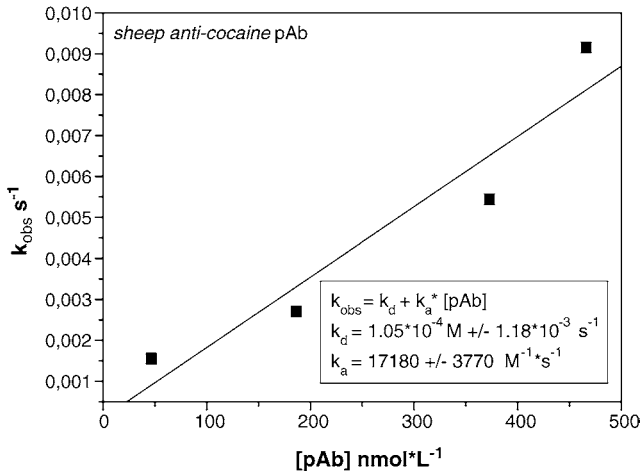

Fig. 5. Plot of k_{obs} resulting from the binding curves of sheep anti-cocaine polyclonal antibody to BZE-DADOO-modified piezoelectric sensor vs. the corresponding concentrations (MW of pAb was assumed to be 150 kDa).

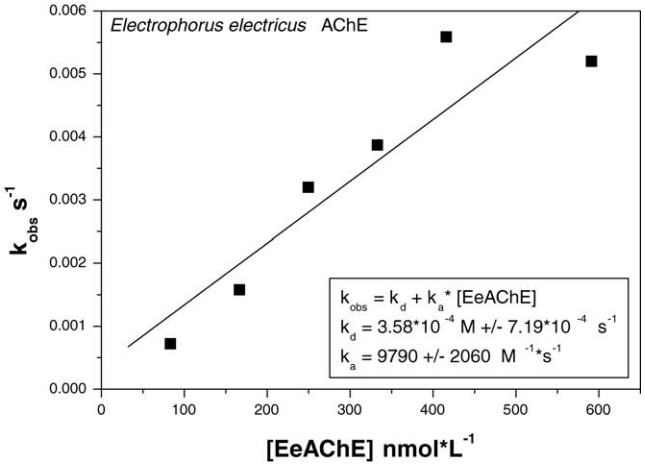

Fig. 6. Plot of k_{obs} obtained by non-linear fitting of the association phase of the binding of *Electrophorus electricus* acetylcholinesterase to the BZE-DADOO-modified piezoelectric sensor vs. the corresponding concentrations (MW of EeAChE was assumed to be 240 kDa).

Table 1
Association and dissocation rate constants

	k_a ($mol^{-1}\,l\,s^{-1}$)	k_d (s^{-1})
Anti-cocaine pAb	$1.71 \times 10^4 \pm 3.77 \times 10^3$	$1.05 \times 10^{-4} \pm 1.18 \times 10^{-3}$
Electric eel AChE	$9.79 \times 10^3 \pm 2.06 \times 10^3$	$3.58 \times 10^{-4} \pm 7.19 \times 10^{-4}$

Table 2
Equilibria constants obtained from kinetic parameters

	K_{eq} ($mol^{-1}\,l$)
Anti-cocaine pAb	162.95×10^6
Electric eel AChE	27.34×10^6

The association rate constant k_a for the polyclonal anti-cocaine antibody was nearly two times higher (k_a $1.71 \times 10^4\,mol^{-1}\,l\,s^{-1}$) as compared with that for *Electrophorus electricus* acetylcholinesterase (k_a $9.79 \times 10^3\,mol^{-1}\,l\,s^{-1}$). The dissociation rate constant for the anti-cocaine antibody k_d was nearly two times lower than for EeAChE (pAb k_d, $1.05 \times 10^{-4}\,s^{-1}$; EeAChE k_d, $3.58 \times 10^{-4}\,s^{-1}$).

The kinetic rate constants for EeAChE correspond well with the previous results obtained with AChE of another origin [9]. On the other hand, the previously published results [6] showed a higher affinity of the strong inhibitor – paraoxon to BChE.

The determination of cocaine was performed in a competitive format. A fixed concentration of $14\,\mu g\,ml^{-1}$ of the antibody was first pre-incubated with sample containing the cocaine for 180 s, then the pre-incubated mixture was allowed to interact with the BZE-DADOO-modified sensor. The binding curves obtained in this way are shown in Fig. 7. As can be seen, $100\,pmol\,l^{-1}$ of cocaine caused a significant decrease of response compared to the maximum response in the absence of cocaine.

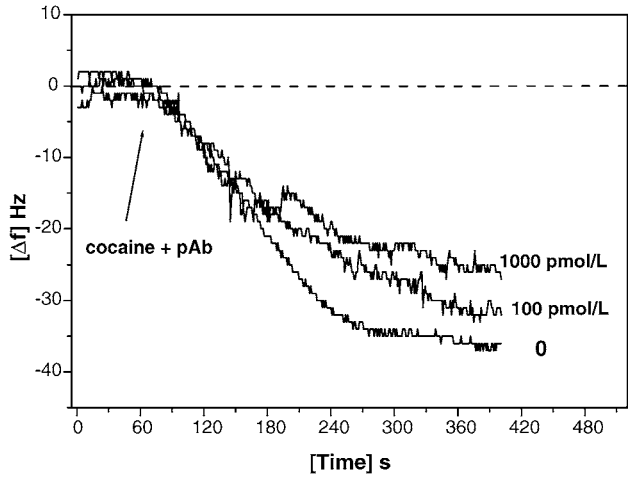

Fig. 7. Binding curves from the competition experiment – binding of pAb pre-incubated with free cocaine (concentrations indicated near the traces).

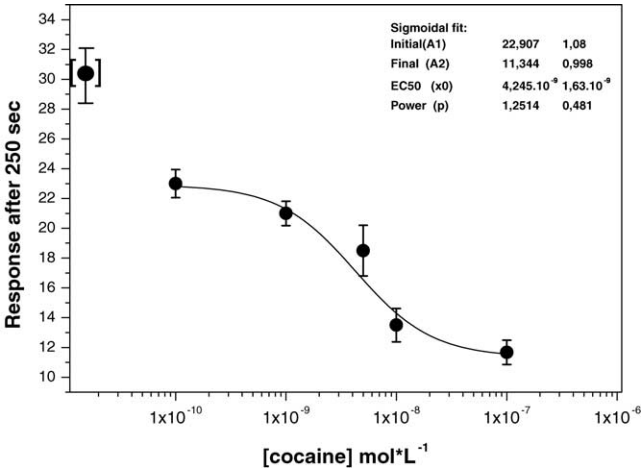

Fig. 8. Calibration curve for competitive assay for cocaine. Average relative error of assay: 7.1% (n = 3, for each concentration of cocaine).

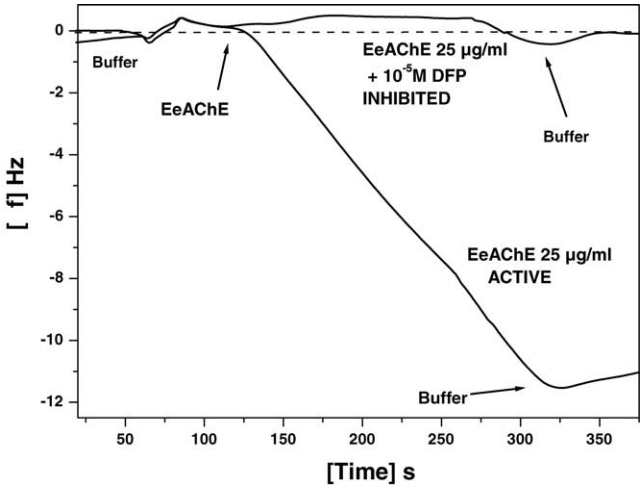

Fig. 9. Binding curves comparing the binding of enzymatically active EeAChE and inactive (inhibited by DFP) EeAChE to immobilized BZE-DADOO (indicated near the traces).

As the signal, the change of frequency measured after 250 s of binding was used (Fig. 8).

Binding of the enzymatically active and fully inhibited EeAChE forms is compared in Fig. 9 . Enzyme with full activity binds to BZE-DADOO on the surface, leading to the saturation value of 11.15 Hz, whereas the addition of the inhibited enzyme results in a minimal frequency decrease (0.17 Hz). This corresponds with the fact that cocaine is a very weak substrate for cholinesterase, i.e. it binds mainly into the catalytically active site and is only very slowly cleaved (k_{cat} of 0.0651 s^{-1}).

4. Conclusions

In this study, the piezoelectric sensor technology was successfully used for monitoring affinity interactions between anti-cocaine antibodies and the immobilized cocaine derivative BZE-DADOO. The determination of cocaine using a competitive scheme is described. The same cocaine derivative – a reversible inhibitor of cholinesterase – was used for binding cholinesterase to the sensor surface. Using non-linear regression – kinetic parameters of both interactions have been determined. These parameters indicate a significantly higher affinity of pAb to cocaine than EeAChE.

Cocaine was determined in water with LOD up to 100 pmol l^{-1} using antibodies. The interaction between *Electrophorus electricus* acetylcholinesterase and its substrate cocaine was evaluated. In summary, the piezoelectric mass-sensitive crystals showed the ability to characterize interactions between different binding partners like antigen – antibody and enzyme – substrate. In future investigations, the detection of cholinesterase inhibitors based on the competitive principle will be optimized and evaluated on real samples.

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