# PERFORMANCE OF THE AMPEROMETRIC BIOSENSOR WITH IMMOBILIZED BUTYRYLCHOLINESTERASE IN ORGANIC SOLVENTS

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The disposable electrochemical sensors with platinum composite electrode were successfully used for amperometric measurement of thiocholine produced from butyrylthiocholine by enzyme hydrolysis with butyrylcholinesterase (BChE, EC 3.1.1.8). The optimum potential was +500 mV vs the internal Ag/AgI/iodide (1 mmol/l) reference electrode. BChE was crosslinked with albumin and glutaraldehyde over the working electrode. Thus obtained biosensor was tested in various organic solvents (values of the hydrophobicity parameter log *P* from –1 to 5), no loss of activity was detected after a 5 min incubation in the solvents with log *P* greater than 2.5. The inhibition of the biosensor with paraoxon was studied in water, toluene, hexane, heptane and nonane. The extent of inhibition increased with solvent hydrophobicity (toluene > hexane > heptane), the inhibition obtained in water was similar as in heptane. Lower response than expected was obtained in nonane. The liquid–liquid extraction of paraoxon from aqueous samples to heptane provided significant improvement of sensitivity of the method. By this method, the lowest detected concentration of paraoxon was 0.03 μmol/l, resulting in the 20% inhibition of the biosensor signal after 5 min preincubation in the heptane extract.

**Key words:** Amperometric biosensor; Cholinesterase.

The enzyme processes involving non-polar reaction components often proceed quite well even in non-aqueous environments, which could be conveniently employed both for biocatalytic and bioanalytical purposes. The first publication on the activity of chymotrypsin in organic solvent appeared some 30 years ago[1]. The enzyme catalysis in non-aqueous environments utilizes increased solubility of substrates, improved thermostability of enzymes and changes in thermodynamic equilibrium to enhance biotechnological processes[2–4]. As it could also be advantageous to work in mixed solutions, the emulsions of water in organic phase or in the opposite way were investigated[5]. The biocatalytic sensors operating in non-aqueous phase followed quickly[6] and their number rapidly increases[7,8]. At first, the determination of analytes which act as substrates for the immobilized enzyme prevailed. A wide group of biosensors employed immobilized tyrosinase for determination of various phenols after extraction from water to organic solvent or directly by dissolving the hydrophobic sample – olive oil – in the organic phase[6,9]. The latter approach was used also for the determination of cholesterol in butter using

cholesterol oxidase[10]. Hydrogen peroxide and organic hydroperoxides were determined using peroxidase sensors operating in various solvents[11], even performance of the mediated glucose oxidase biosensor was investigated in acetonitrile[12].

The effects of organic solvents on the inhibition of enzyme reactions were studied[3]. The inhibiting properties increased with decreasing solubility of the inhibitor in water. Recently, amperometric biosensors for thioureas using peroxidase[13] and tyrosinase[14] in hexane appeared. This approach was studied also for the acetylcholinesterase-based sensor[15] intended for the determination of organophosphate pesticides. In fact, this type of measurement could be promising for the analysis of this kind of pesticides in environmental samples (water, soil, food). As the first step of analysis, the extract of pesticides from sample in organic solvent (hexane, dichloroethane) is prepared, the vacuum drying follows and finally the analysis is completed using either liquid chromatography[16] or inhibition test kit based on free cholinesterase[17].

In this work, the previously developed disposable biosensor based on butyrylcholinesterase[18] is tested in various non-polar solvents and the determination of a model pesticide paraoxon in aqueous phase and in organic extracts is compared.

## EXPERIMENTAL

*Material*. Butyrylcholinesterase (BChE, specific activity 16 μcat/mg) and paraoxon were obtained from Sigma (Saint Louis, U.S.A.). Glutaraldehyde (25% solution) was purchased from Reanal (Budapest, Hungary), hexane and nonane from Loba Chemie (Vienna, Austria), and heptane from Merck (Darmstadt, Germany). All other chemicals were supplied by Lachema (Brno, Czech Republic) and the Millipore quality distilled water was used throughout. The screen-printed electrochemical sensors – ceramic strips 7 × 25 mm containing the platinum-based working electrode ( ∅ 1 mm) surrounded by the silver-based reference electrode (area 5.5 $mm^2$) were purchased from Krejci Engineering (Tisnov, Czech Republic), for detailed description see ref.[19].

*Preparation of biosensors*. The enzyme layer was prepared by drop coating the working electrode with 1 μl of the previously optimized mixture containing BChE (600 ncat/ml), bovine serum albumin (3.5 mg/ml) and glutaraldehyde (0.8 mg/ml) in 50 mM phosphate buffer, pH 7.0. The sensors were immediately placed in refrigerator, allowed to dry overnight and then stored in dry state at 4 °C.

*Measuring procedures*. For experiments, the amperometric detector ADLC 2 (Laboratorni pristroje, Prague, Czech Republic) was connected through a 12-bit A/D converter (Datik, Brno, Czech Republic) to computer. An own software (LabTools) running under Windows was used for data display, storage and evaluation.

All experiments were performed at laboratory temperature (25 ± 2 °C), a thermostat was not used. For amperometric measurements, the dry biosensor in a horizontal position was connected to the detector, the applied potential was +500 mV vs the internal Ag pseudoreference electrode. The measurement started by dropping 50 μl of substrate solution (1 mM butyrylthiocholine iodide, BTChI, dissolved in 50 mM phosphate buffer, pH 7.0) and then the trace of current was recorded for 200 s. The signal was calculated as a mean value of current obtained during the time interval from 150 to 200 s (100 points, delay interval 0.5 s), the addition of substrate represented always the beginning.

To evaluate the effect of inhibitors, the initial signal $S_0$ of the biosensor was determined, then the biosensor was placed in a closed vessel containing 1 ml of the sample (solution of paraoxon in either aqueous or organic solvent) and incubated for 5 min. Then, the biosensor was washed, allowed to dry

(3 min) and the final signal $S_E$ was measured again. The inhibition $I$ (%) was calculated from the decrease of signal:

$$I = 100(1 - S_E / S_0) \,. \qquad (1)$$

## RESULTS AND DISCUSSION

### *Characterization of Biosensors*

In our previous works, the BChE biosensors employed a chemically modified graphite composite electrode as the transducer[21]. Butyrylthiocholine as substrate was hydrolyzed by cholinesterase and the produced thiocholine was anodically oxidized. The electrode layer was prepared using acetylcellulose as a binder and cobalt phthalocyanine as the modifying agent. Unfortunately, this layer was not suitable for operation in non-aqueous environment as the layer swelled and became mechanically damaged. For this reason, we decided to employ a platinum electrode prepared by screen printing. It was shown that the oxidation of thiocholine at a platinum metal electrode is achieved at potentials close to 0.4 V vs the common Ag/AgCl reference[15]. The platinum electrode employed here is not a pure metal electrode, some amount of the binding material from the original printing paste is present, too.

To study the behaviour of biosensors, the response to the addition of a drop of substrate was recorded for both bare and BChE-modified sensors using increasing potential of the working electrode (Fig. 1). The addition of butyrylthiocholine iodide in phosphate caused two effects. At first, the potential of the reference electrode – silver layer – is established. In fact, in the presence of iodide anions, the silver layer is coated with silver iodide and the reference potential is then given by the Ag/AgI/iodide (1 mmol/l) system. Under the experimental conditions, this internal reference is some 150 mV below the standard Ag/AgCl/KCl (3 mol/l) electrode. Of course, from the electrochemi-

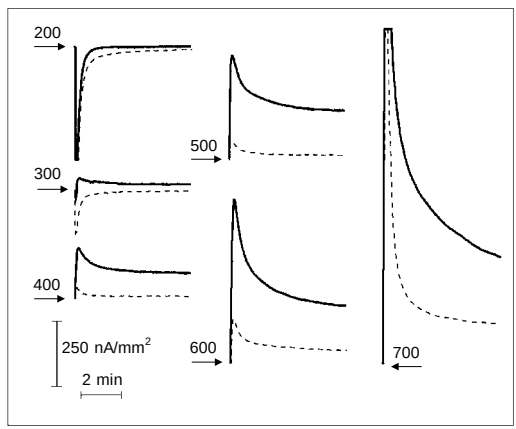


FIG. 1
Traces of current recorded after applications of 50 μl drops of substrate (1 mmol/l BTChI) on dry bare (dashed lines) and BChE-modified (full lines) sensors. The horizontal arrows represent the zero level of current, the numbers close to them indicate the potentials applied to the working electrodes

cal point of view, this reference is not perfect, but it serves quite well for the short-time recording of the biosensor current. All values of potentials are indicated against the internal reference electrode.

The second process – the enzyme reaction and the following oxidation of the product – is much more interesting. As can be seen, in the absence of BChE and thus in the absence of thiocholine (Fig. 1, dashed lines), a reasonably stable background current was obtained within less than 2 min since the addition of substrate. A significant increase of the background occurred for potentials above 500 mV, which was caused by the competing anodic oxidation of the present iodide anions at the electrode. With immobilized BChE (Fig. 1, full lines), a steady state was obtained within 2 min for potentials below 600 mV. The useful signal (difference between full and dashed lines) was higher with increasing potential on the working electrode. For practical measurements, the potential of 500 mV was chosen; under this conditions, a high signal is obtained and the interference of iodide is still negligible.

In the next part, the effect of several organic solvents on the activity of the BChE biosensor was tested. The change of the signal of the biosensor after a 5 min incubation in the given solvent was determined. The obtained results (relative signal $S_{rel}$) were plotted against the log *P* values for the given solvents (Fig. 2). The log *P* (*P* is water/octanol partition coefficient) parameter characterizes polarity of solvents, the numeric values were taken from ref.[22]. A characteristic shape of the curve was obtained. Evidently, solvents with log *P* below 2.5 are not suitable for the BChE biosensor as the signal decreases significantly even in the absence of any inhibitor. To our best knowledge, no data are available in literature on the effect of organic solvents on BChE, however, a comparison could be made with acetylcholinesterase (AChE). For soluble enzyme, the water-miscible organic solvents act as inhibitors[23]. A significant inhibition was observed for free AChE in solvents of log *P* below 3; the immobilization of AChE in photocrosslinked poly(vinyl alcohol) provided significantly improved sta-

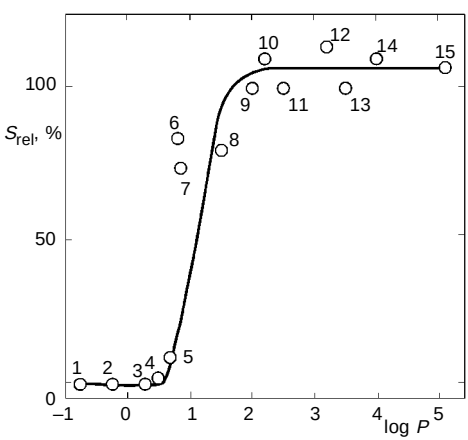


FIG. 2
Signal of the BChE biosensor after incubation in organic solvents. The relative signal with substrate in phosphate buffer determined after 5 min incubation of the biosensor in the given solvent ($S_{rel}$, 100% represents always the initial signal) is plotted against the partition coefficient (log *P*). The following solvents were used: 1 methanol, 2 ethanol, 3 propanol, 4 tetrahydrofuran, 5 ethyl acetate, 6 butanol, 7 diethyl ether, 8 cyclohexanol, 9 chloroform, 10 amyl acetate, 11 toluene, 12 cyclohexane, 13 hexane, 14 heptane, 15 nonane

bility, solvents with log *P* close to 1 did not cause decrease of activity[15]. The immobilization of AChE evidently improved the tolerance to less hydrophobic organic solvents, i.e. those which could solubilize the water which was originally present inside the biolayer. In our BChE sensor, crosslinking with glutaraldehyde is used for immobilization, which provides relatively thin enzyme layers; the content of activity in the layer for detection of inhibitors should not be high to provide good sensitivity.

*Effect of Solvents on Inhibition with Paraoxon*

Several solvents which did not decrease the activity of BChE were used for construction of inhibition curves for the pesticide paraoxon, which is widely used as a model organophosphate compound. A stock solution of paraoxon was made in methanol and it was used to prepare solutions of variable paraoxon concentration in the given solvents; the content of methanol was always below 0.2% in the final mixture. The inhibition curves were subsequently measured (Fig. 3) as a decrease of the signal of the biosensor with substrate after a 5 min incubation in the solvent containing paraoxon. The same experiment was also performed using distilled water. Similar sensitivities were obtained for inhibitions performed using either water or heptane, the other solvents provided lower responses. To characterize the effect of the polarity of the environment on the inhibition, the sensitivities were expressed as $pI_{50}$ (the negative logarithm of the concentration of paraoxon providing 50% inhibition during the 5 min interval, Table I). For toluene, hexane and heptane, the increasing hydrophobicity resulted in improved sensitivity, heptane approached the value for water (included in Table I for comparison). In nonane, which was the most hydrophobic solvent studied, the sensitivity was the lowest. The reason for this could be the worse contact between this solvent and BChE molecule. The sensitivity does not depend only on the hydrophobicity of the environment, but the structure and functional groups could play significant role, too.

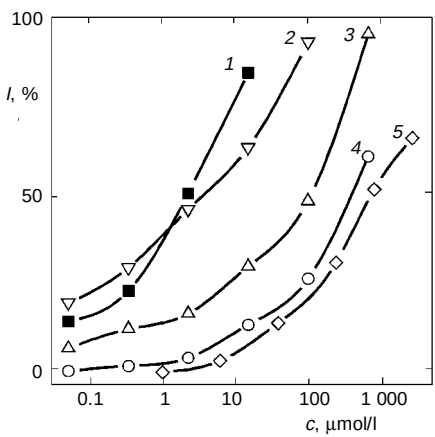


FIG. 3
Inhibition curves for paraoxon determined as a percentual decrease of signal (*I*, %) resulting from a 5 min incubation of the BChE biosensor in the solution of paraoxon (concentration *c*) in the following solvents: *1* water, *2* heptane, *3* hexane, *4* toluene and *5* nonane. The curves were drawn through the experimental points (spline procedure) for a simplified view

With AChE based sensor[15], much better responses were obtained for aromatic and especially aliphatic hydrocarbons (toluene, hexane) than for other compounds (tetrachloromethane, butyl acetate) exhibiting quite comparable log *P* values. On the other hand, the response obtained in tridecane was much smaller than in octane and hexane.

Finally, the possible analytical application of this biosensor was tested. We tried to combine the extraction of pesticides from water with biosensor analysis. The solutions of increasing paraoxon concentration in water were prepared and used directly for analysis (Fig. 4, curve *1*). On the other hand, these solutions were mixed with heptane (2 ml of heptane per 20 ml of the aqueous sample) and vigorously shaken for 10 min. After separation of phases, the heptane solution was analyzed using the biosensor (Fig. 4, curve *2*). The horizontal axis in Fig. 4 represents always the original concentration of paraoxon in aqueous phase. Evidently, the extraction of paraoxon from water to heptane

TABLE I
The effect of polarity of the solvent (log *P*) on the biosensor sensitivity to paraoxon characterized as a concentration (mol/l) required for 50% inhibition ($pI_{50}$, obtained after a 5 min incubation)

| Solvent | log *P* | $pI_{50}$ |
|---|---|---|
| Water | – | 5.63 |
| Toluene | 2.5 | 3.40 |
| Hexane | 3.5 | 3.96 |
| Heptane | 4.0 | 5.42 |
| Nonane | 5.1 | 3.11 |

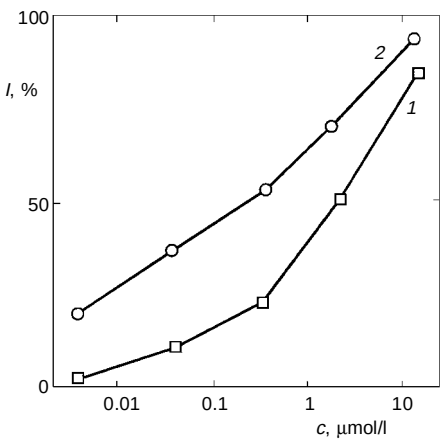


FIG. 4
Calibration curves for paraoxon in water obtained by incubation of the BChE biosensor either directly in the aqueous sample (*1*) or in the heptane extract (*2*) which was prepared from the original sample by 10 min extraction (heptane to water volume ratio 1 : 10)

increased significantly the sensitivity. When using the extraction procedure, approximately 10 times higher signal was obtained for the given original aqueous sample.

## CONCLUSIONS

It was demonstrated that the composite platinum electrode prepared by thick film technology is well suited for the amperometric measurement of thiocholine and thus it could serve as a transducer for biosensors with cholinesterases as biorecognition elements. The prepared biosensors were successfully applied for detection of paraoxon by means of inhibition in organic phase. The best results – highest inhibitions – were achieved using heptane as a solvent. The sensitivity of analysis was significantly improved using preconcentration of paraoxon from aqueous samples to heptane. In future work, the liquid–liquid extraction tested here could be replaced by the solid phase extraction to achieve even better sensitivity. In future, the developed bioanalytical method for screening of pesticides using disposable biosensors operating in organic phase will be verified on real samples (water, soil and food products).

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