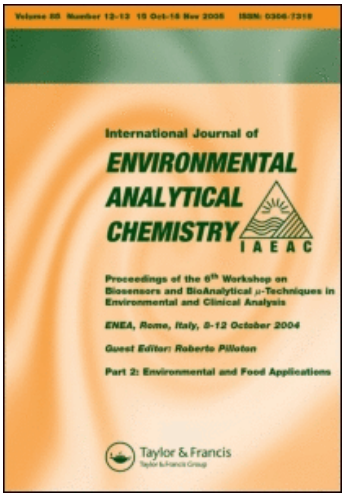

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A Four-Channel Electrochemical Immunosensor for Detection of Herbicides Based on Phenoxyalkanoic Acids

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A FOUR-CHANNEL ELECTROCHEMICAL IMMUNOSENSOR FOR DETECTION OF HERBICIDES BASED ON PHENOXYALKANOIC ACIDS

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An electrochemical immunosensor combining three monoclonal antibodies on the same measuring element produced by screen-printing was constructed for the semiquantitative group-specific detection of herbicides belonging to phenoxyalkanoic acids. The combination of different monoclonal antibodies covalently immobilized on three working electrodes and one additional electrode containing immobilized albumin provided a more reliable detection of phenoxyalkanoic acids. Peroxidase-2,4-dichlorophenoxyacetic acid conjugate was used as a tracer suitable for all the antibodies employed in a competitive immunoassay. Several herbicides were used to characterize the immunosensor. The developed immunosensor was tested on samples of surface water spiked with different herbicides and binary mixtures of herbicides. The proposed approach seems feasible for rapid screening of large series of samples for the presence of herbicides.

Keywords: Electrochemical immunosensor; Multichannel biosensor; Herbicides; Phenoxyalkanoic acids

INTRODUCTION

The increasing public concern on environmental protection and recent legislative requirements have stimulated increasing demand on rapid and economical analytical methods for determination of toxic compounds including pesticides. The current needs for more intensive screening of pesticide residues are usually satisfied with immunochemical methods represented by ELISA [1,2]. So far, most of the immunoassays is directed towards a single target analyte with no or limited response to similar compounds. Though this is the biggest advantage of bioanalytical methods, sometimes, the screening of a group of similar compounds will be preferable. Several biosensor systems are capable to provide group-specific response; e.g. organophosphates can be detected using devices based on the inhibition of cholinesterase [3], phenolic compounds are recognized by tyrosinase [4]. In the area of immunoassays, the group-specific

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response is rather rare, as it is not easy to produce the appropriate antibodies. Some examples include antibodies to the common moiety of organophosphorus pesticides [5]. For screening tests based on immunochemical assays, the detection of a whole group of related toxic compounds is highly desirable; in this way, potentially harmful samples could be identified with lower cost and workload. The multianalyte immunochemical systems should preferably relay on the advanced biosensor systems [6]; some examples suggested so far include, especially, optical immunosensors [7,8]. Further extension of the multisensing concept includes combination with pattern recognition chemometric techniques; however, antibodies exhibiting sufficient cross-reactivity towards individual members of a group are required [9].

Electrochemical immunosensors combine high sensitivity of the transducer with excellent specificity of immunochemical interactions [10]. Research in our laboratory has focused for many years on construction of electrochemical immunosensors for determination of the herbicide 2,4-dichlorophenoxyacetic acid (2,4-D) using monoclonal antibodies; the results obtained by us as well as by others were compared recently [11]. So far, the focus was directed only towards the most important compound 2,4-D. In this article, the electrochemical immunosensor combining three antibodies with different specificities was constructed in order to allow detection of a broader spectrum of herbicides belonging to the group of phenoxyalkanoic acids.

EXPERIMENTAL

Materials Cystamine dihydrochloride and 2,4-D were obtained from Fluka (Buchs, Switzerland). Glutaraldehyde (25%) was from Reanal (Budapest, Hungary). *N*-hydroxysuccinimide (NHS), dicyclohexylcarbodiimide (DCCD) and peroxidase (EC 1.11.1.7, POD, from horseradish, 100 IU/mg) were supplied from Sigma (St. Louis, MO, USA). Herbicides (all in purity better than 98%) 2-(2,4-dichlorophenoxy)-propionic acid (2,4-DP), 2,4,5-trichlorophenoxyacetic acid (2,4,5-T), 2-methyl-4-chlorophenoxyacetic acid (MCPA), 2-methyl-4,6-dichlorophenoxyacetic acid (MDPA), monoclonal antibodies (MAb) anti-MCPA clone C2C3, and anti-2,4-D clones E2/G2 and G5E10, were all kindly provided by Dr. Milan Fránek (Veterinary Research Institute, Brno, Czech Rep.). MAbs were provided as IgG fractions prepared from the mouse ascites fluids by precipitation with ammonium sulphate [12]. All other chemicals were obtained from Lachema (Brno, Czech Rep.) and Millipore system deionized water was used for all experiments.

Biosensors A four-channel electrochemical sensor was produced according to our proposal at Krejčí Engineering (Tišnov, Czech Rep.). The design of the sensor is shown at Fig. 1, it consists of four circular gold-based working electrodes (1 mm diameter) and a common silver counter electrode (4×2 mm), printed on a ceramic support (alumina), the contacting paths are covered by an insulation layer. At the beginning of immobilization, the sensor was carefully washed in acetone for 30 min. The working electrodes were then coated with 1 μL of an aqueous cysteamine solution (20 mg/mL) for 2 h at room temperature. After washing with water, the free amino groups on the surface were activated with glutaraldehyde (3% in water) for 1 h. The individual working electrodes on the sensor were washed with water and coated with 1 μL of the antibodies E2G2, G5E10, C2C3 and one with albumin, all dissolved at protein concentration of 1 mg/mL in phosphate buffer. After incubation overnight, the formed

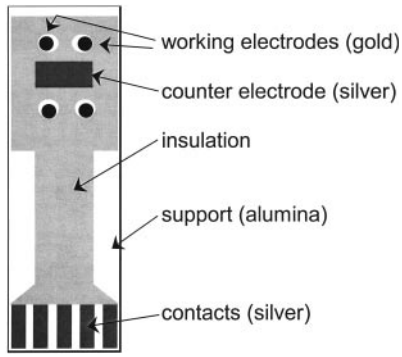

FIGURE 1 Scheme of the screen-printed sensor consisting of four working and common reference electrodes, overall dimensions 38 × 12 mm.

Schiff base was stabilized by reduction with 50 mM sodium cyanoborohydride in water for 30 min. Thus obtained biosensors were stored in dry conditions in a refrigerator.

Tracer synthesis The 2,4-D-POD conjugate was produced as follows. 2.3 mg of NHS and 4.1 mg of DCCD were dissolved in 4 mL of dimethylformamide (DMF) and mixed with 2 mL of 2,4-D (4.5 mg) solution in DMF. After the 4-h incubation at room temperature, 300 μL of the mixture containing NHS-activated 2,4-D were mixed with peroxidase (5 mg) dissolved in 2 mL 50 mM phosphate buffer pH 7.4. The conjugation proceeded for 30 min at room temperature and the resulting 2,4-D-POD tracer was dialyzed for 2 days against phosphate buffer (20 mM, pH 7.4), diluted to protein concentration of 1.5 mg/mL and stored frozen in small aliquots.

Measuring procedure The sample (either water or standard solution of herbicide in distilled water) was mixed with the POD-2,4-D tracer (1.5 μg/mL final concentration) and dropped on a horizontally fixed sensor. The incubation proceeded for 30 min and the immunosensor was finally washed with water. The electrochemical response was measured with the four-channel sensor immersed in a beaker containing 10 mL of 50 mM sodium acetate buffer, pH 4.5, stirred at 300 rpm. The four working electrodes were polarized at −50 mV *vs* the common silver pseudoreference, a custom made four-channel potenciostat (constructed at Krejčí Engineering) was controlled through a multipurpose card containing A/D (14 bit) and D/A (12 bit) converters (Haal, Brno), an own program LabTools was used for measurements. After stabilization of the background current (typically 1 min), potassium iodide (1 mM) was added followed by hydrogen peroxide (2 mM, final concentrations). The change of current due to the addition of peroxide was recorded after 1 min. Typical traces are shown at Fig. 2 for the electrode containing POD-labeled immunocomplex and reference blank. No regeneration of immunosensors was carried out and each sensor was used only once.

RESULTS AND DISCUSSION

Characterization of the Immunosensor

The construction of the multichannel immunosensor employed antibodies immobilized at three working electrodes, the remaining working electrode was modified similarly with albumin in order to allow correction for possible nonspecific signals. The covalent

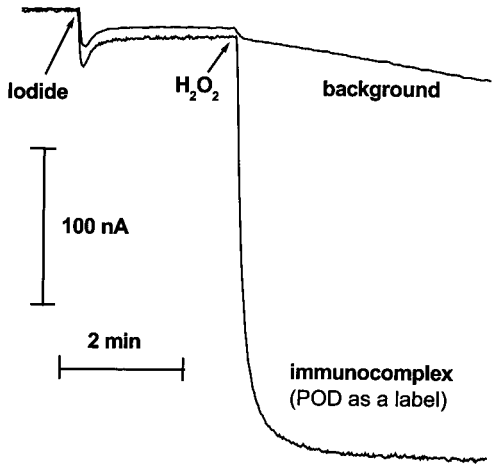

FIGURE 2 Examples of electrochemical evaluation of the immunoreaction. The upper trace (background) was obtained in the absence of any immunocomplex or peroxidase; the lower curve was obtained after formation of the peroxidase-labeled immunocomplex on the surface of electrode. The final concentrations of iodide and hydrogen peroxide were 1 and 2 mM, respectively.

immobilization based on a self-assembled monolayer of cystamine on gold surface appeared to be quite robust with respect to stability and reproducibility of production [11]. The 2,4-D-POD conjugate was chosen as the tracer and it was able to form immunocomplexes with all the employed antibodies. The peroxidase activity bound to the electrode in the immunocomplex was measured using iodide and hydrogen peroxide as substrates; the enzymatically formed iodine was determined by cathodic reduction. A high response was obtained in the presence of the immunocomplex, and a steady-state current established quite quickly (Fig. 2). The signal was measured 1 min after addition of both substrates in order to minimize the slowly appearing non-enzymatic signal (Fig. 2, trace "background") resulting from the spontaneous oxidation of iodide by hydrogen peroxide. Though many other substrates were tested for the electrochemical determination of peroxidase [13,14], the small molecule of iodine can more easily diffuse through several protein layers covering the electrode. Consequently, it provides higher currents in real electrochemical immunoassays.

Detailed calibration curves for two chosen analytes 2,4-D and MCPA were constructed in concentration ranges from 0.01 to 1000 μg/L; the responses from the four measuring channels were always recorded simultaneously in stirred conditions. This format was adopted as the most convenient for initial studies, though in future the whole assay can be directly performed on the immunosensor using the pulsed approach reported previously [13]. The measured currents I from the individual working electrodes with antibodies were used to calculate the relative responses:

$$B_{\mathrm{rel}} = (I - I_{\mathrm{BSA}})/(I_0 - I'_{\mathrm{BSA}})$$

I_0 represents the maximum response obtained in the absence of any free herbicide, I_{BSA} and I'_{BSA} were the currents obtained at the corresponding working electrodes coated with albumin. The channel with albumin allowed subtracting small responses due to

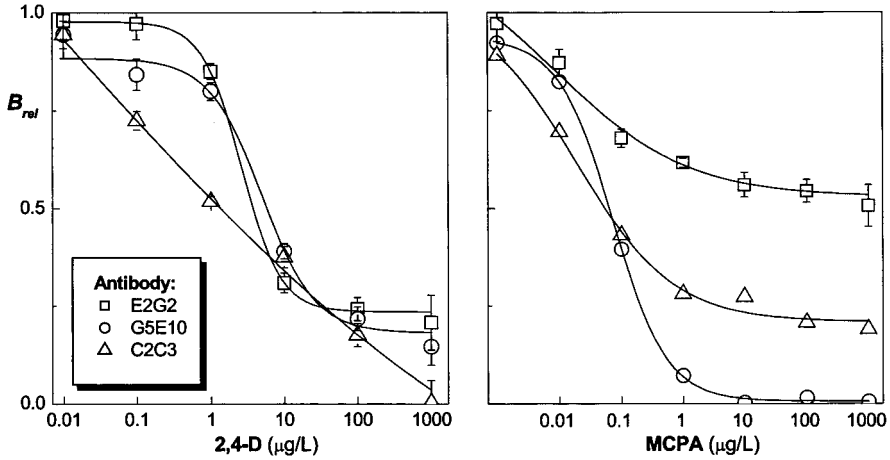

FIGURE 3 Calibration curves for the herbicides 2,4-D (left) and MCPA (right) using the multichannel immunosensor with three different MAbs and a reference zone with BSA. The parameter B_{rel} was obtained as the relative response of the given sensing zone in the presence and absence of herbicides, both corrected for nonspecific signal obtained at the zone with BSA.

potential nonspecific adsorption of the tracer (this was quite negligible) and also the spontaneous nonenzymatic oxidation of iodide (compare Fig. 2). In this way, three values of B_{rel} corresponding to three MAbs are obtained from each measurement.

As can be seen from, thus, generated calibration curves (Fig. 3), three different types of dependences were obtained. The combinations 2,4-D plus antibodies E2G2 and G5F 10, and MCPA with antibody G5E10 provided quite steep dependences as expected for typical immunoassays limited by the kinetics of the immunochemical interaction. MCPA was not able to compete efficiently with the tracer (2,4-D-POD) for the binding sites of antibodies E2G2 and C2C3 providing limiting values of B_{rel} near 0.50 and 0.20, respectively. This is rather surprising as the antibody C2C3 was selected for the assay of MCPA, however, it was previously used in a different immunoassay format (M. Fránek, unpublished results). Finally, the response of 2,4-D obtained with electrodes containing immobilized MAb C2C3 was linear (B_{rel} *vs* $\log c_{2,4\text{-D}}$) over several concentration ranges; such behavior was observed previously and it is considered typical for immunosensors limited by the mass transfer process [11,13]. The calibration points at Fig. 3 were approximated with the sigmoidal logistical equation:

$$B_{rel} = A_2 + \frac{A_1 - A_2}{1 + (c/c_0)^p]}$$

The values of parameters obtained by nonlinear regression are summarized in Table I and were later used to calculate concentrations (as 2,4-D equivalents) from the experimental B_{rel} values using the inversed equation.

The cross-reactivity of the three MAbs towards several phenoxyalkanoic acids was tested with herbicides dissolved at 1 μg/L in distilled water (Table II). At this concentration, most herbicides were able to provide decrease of binding of the tracer even higher than 2,4-D. This is surprising, because the published [12] cross-reactivities of the antibodies E2G2 and G5E10 were much lesser, however, for antibodies in

TABLE I The parameters (values and standard deviations) of the sigmoidal logistical equations fitted to the experimental calibration curves from Fig. 3

Antibody	*Herbicide*	A_1	A_2	c_0 (μg/L)	P
E2G2	2,4-D	0.976 ± 0.032	0.235 ± 0.029	2.62 ± 0.51	1.64 ± 0.26
	MCPA	1.18 ± 0.30	0.528 ± 0.037	0.008 ± 0.020	0.40 ± 0.17
G5E10	2,4-D	0.884 ± 0.037	0.181 ± 0.030	4.99 ± 0.97	1.21 ± 0.23
	MCPA	0.9348 ± 0.0092	0.0060 ± 0.0045	0.0724 ± 0.0031	1.002 ± 0.043
C2C3	2,4-D	2.3 ± 3.9	−0.5 ± 1.2	0.02 ± 0.26	0.13 ± 0.21
	MCPA	1.03 ± 0.11	0.208 ± 0.021	0.018 ± 0.011	0.55 ± 0.12

TABLE II Comparison of responses expressed as relative decrease of the binding of the tracer, $((1-B_{rel})/(1-B_{rel,2,4-D}))$ 100%, obtained with different herbicides from the group of phenoxyalkanoic acids (B_{rel} determined for herbicide concentration 1 μg/L)

Herbicide	*Immobilized antibody*		
	E2G2	*G5E10*	*C2C3*
2,4-D	100	100	100
MCPA	154	176	56
2,4,5-T	169	279	108
2,4-DP	338	148	146
MDPA	515	528	49

TABLE III Results of the analysis of spiked distilled water samples for the three antibodies, calculated as 2,4-D equivalents in μg/L. The concentration values (means from 3 measurements) were obtained using calibration curves for 2,4-D from Fig. 3 and the estimated standard deviations are shown

Added herbicide (1 μg/L)	*Found 2,4-D equivalents* (μg/L) *based on antibody*		
	E2G2	*G5E10*	*C2C3*
2,4-D	0.93 ± 0.15	0.83 ± 0.19	0.58 ± 0.11
2,4-DP	2.87 ± 0.33	1.07 ± 0.13	0.73 ± 0.23
2,4,5-T	1.50 ± 0.27	3.11 ± 0.58	0.28 ± 0.12
MCPA	1.12 ± 0.12	1.23 ± 0.21	0.151 ± 0.043
MDPA	6.95 ± 0.78	28.5 ± 5.7	0.048 ± 0.014

solution and in a different ELISA format. As the differences in responses obtained within individual antibody–herbicide combinations were rather small (with the exception of MCPA and MDPA exhibiting much lower affinity to MAb C2C3), the chemometric analysis was not applied as similar patterns of affinities would prevent more detailed characterization of unknown samples with respect of the type of present herbicides. However, the combination of antibodies appeared promising for the proposed group-specific detection of phenoxyalkanoic acids.

For practical reasons, it was decided to express concentrations of "unknown" herbicides in samples as 2,4-D equivalents using calibration curves presented for individual antibodies at Fig. 3. The apparent 2,4-D contents for the individual pesticides in distilled water were obtained and presented in Table III. In an ideal case, all the values should be equal to 1 μg/L, as was the concentration of herbicides

added to distilled water. The determined values reflect different specificities of the three antibodies and cross-reactivities of antibodies against individual herbicides. The values obtained with MAbs E2G2 and G5E10 were acceptable (considering the expected group-specific response) ranging from 0.83 to 3.11 μg/L for all herbicides with the exception of MDPA, which was rather 'overestimated' due to the higher affinity to the immobilized antibody. On the other hand, MAb C2C3 significantly 'underestimated' MCPA and MDPA. For practical application on real samples of water, the sample should be considered as positive when at least one channel indicates B_{rel} below some selected threshold value; such samples should be then reanalyzed using reference methods.

Evaluation on Real Samples of Water

The sample of ground water (S1) was taken from a small creek near Uherské Hradiště (Czech Rep.) and the sample of well water (S2) was taken from a natural spring located near an agriculture field. The Sample S2 provided measurable analytical signal indicating presence of some target compounds, the detected level was 0.14 ± 0.04 μg/L of the equivalents of 2,4-D. The Sample S1 did not exhibited any response with the immunosensor and it was selected for spiking experiments. This water was spiked with individual herbicides and with mixtures of two herbicides, always at 1 μg/L, of the total concentration. Thus obtained samples were analyzed with the biosensor and the determined relative bindings of the tracer were used to calculate apparent 2,4-D concentrations using calibration curves from Fig. 3. The results for single herbicides presented in Table IV for spiked real water samples can be compared with similar results obtained with spiked distilled water (Table III). The best agreement between distilled and surface water was achieved in the case of 2,4-DP, where nearly identical values were measured; consequently, no matrix effect of the surface water affected the assay for this herbicide. Quite reasonable correlation was found also for MCPA, the values were 50% higher in the real water with all types of antibodies. Good agreement for 2,4-DP was achieved with MAbs E2G2 and C2C3, the value with MAb G5E10 was 1.5-times lower in the real water. The poorest correlation was obtained for 2,4-D, which provided in the real water values twice, 4 and 10-times higher with MAbs E2G2, G5E10 and C2C3, respectively. From all cases, at least two channels on the immunosensor would correctly identify the sample with added herbicide as positive thus providing useful qualitative information.

Another part of Table IV presents results of analysis of the surface water spiked with mixtures (1 : 1) of two herbicides. Prediction of results for mixtures is not straightforward as the resulting relative binding cannot be estimated as a simple linear combination of the individual contributions due to the nonlinear calibration curves. Nevertheless, acceptable results were measured with the channels containing antibodies E2G2 and G5E10, the only exception being mixture of 2,4-D and MDPA with antibody G5E10; in this case, the observed value of 0.90 μg/L was substantially lower than similar results for the individual compounds (2.57 and 13.7 μg/L for 2,4-D and MDPA, respectively). On the contrary, the mixture 2,4-DP and MDPA with MAb C2C3 provided value of 0.67 μg/L, which is higher than the individual data 0.45 and 0.026 μg/L for 2,4-DP and MDPA, respectively.

TABLE IV Recoveries of different phenoxyalkanoic acid compounds from spiked natural water samples. Results as 2,4-D equivalents in μg/L obtained for water sample spiked either with individual herbicides or with mixtures of two herbicides. The concentration values (means from 3 measurements) were obtained using calibration curves for 2,4-D from Fig. 3 and the estimated standard deviations are shown

Total added herbicides	*2,4-D equivalents based on antibody*		
(1 μg/L)	*E2G2*	*G5E10*	*C2C3*
2,4-D	1.95 ± 0.23	2.27 ± 0.37	4.11 ± 0.94
2,4-DP	2.91 ± 0.65	1.16 ± 0.31	0.52 ± 0.13
MCPA	1.63 ± 0.29	1.49 ± 0.34	0.056 ± 0.015
MDPA	7.3 ± 1.2	12.2 ± 2.3	0.028 ± 0.009
2,4-D + 2,4-DP (1 : 1)	2.12 ± 0.32	1.98 ± 0.29	0.71 ± 0.18
2,4-D + MDPA (1 : 1)	2.21 ± 0.24	0.95 ± 0.19	0.43 ± 0.13
2,4-DP + MDPA (1 : 1)	4.23 ± 0.44	4.32 ± 0.25	0.61 ± 0.17

CONCLUSION

The constructed immunosensor combining three monoclonal antibodies on the same electrochemical measuring system appeared as suitable for the planned semiquantitative group-specific detection of herbicides belonging to phenoxyalkanoic acids. The combination of antibodies provides more reliable detection and decreases the amount of false negative results, which were observed for individual antibodies (mostly C2C3). Unfortunately, the specificities of antibodies towards individual herbicides were not different enough to allow better qualitative characterization of unknown samples using chemometric techniques like pattern recognition; additional antibodies should be tested and also the number of channels should be extended to improve this situation. Regarding the precision of analysis, the observed discrepancies may be partially explained by the differences among individual sensors due to the manual production, which cannot be easily corrected for single-use analytical devices. Nevertheless, the proposed approach seems feasible for rapid screening of large series of samples for the presence of herbicides. The obtained qualitative information on the presence and semiquantitative indication on the concentration of the target compounds would help to rapidly select positive samples for subsequent more complicated and precise analytical methods.

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