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The immunosensors for measurement of 2,4-dichlorophenoxyacetic acid based on electrochemical impedance spectroscopy

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Abstract

Electrochemical impedance spectroscopy (EIS) was evaluated for the direct determination of herbicide 2,4-dichlorophenoxyacetic acid (2,4-D). Specific antibody against 2,4-D was immobilised onto different gold electrodes. Several methods of antibody immobilisation by covalent linkage to modified surface were studied. Self-assembled monolayers formed using thiocompounds as cystamine, 4-aminothiophenol (ATPh), 3,3′-dithiopropionic acid di-(*N*-succinimidyl ester) (DTSP) and 11-mercaptoundecanoic acid (MUA) were chosen for the sensing surface activation. Three different sensor types were tested: screen-printed disc and finger-like structures and interdigitated array (IDA) electrodes produced by lithography. The measurements were carried out in a stationary arrangement, and the reaction between hapten and the immobilised antibody was observed online. Changes of impedance parameters were evaluated, and the best immobilisation technique (using 4-aminothiophenol) was chosen for further measurements. Impedance changes due to immunocomplex formation were evaluated, and the possibility of direct monitoring of 2,4-D binding to the antibody was demonstrated at a fixed frequency. For the strip sensor, the calibration curves were constructed in concentration range from 45 nmol l^{-1} to 0.45 mmol l^{-1} of 2,4-D.

Keywords: Electrochemical impedance spectroscopy; 2,4-dichlorophenoxyacetic acid; Screen-printed sensor; IDA electrodes; Immunosensor

1. Introduction

Electrochemical impedance spectroscopy (EIS) is a sensitive technique, which monitors response of the studied system to the application of a periodic small amplitude AC signal. Measurements are carried out at different frequencies. Analysis of the system response contains information about the interface, its structure and occurring reactions. This technique does not lead to the identification of the chemical bonds or of the intermediates; however, information on the reaction rates occurring at the electrochemical interface can be obtained and provide characterisation of the intermediates [1].

EIS immunosensors were utilized for determination of antigen–antibody interactions. In many cases, polypyrrole was used as an integrated recognition and transduction system for the immobilisation of biorecognition element. The example is an immunosensor for detection of luteinising hormone and for DNA hybridisation discrimination able to differentiate single- and double-stranded DNA [2]. Another immunosensor used avidin–biotin for antibody immobilisation [3] allowing design of a highly reproducible and stable device. A flow injection system using two electrodes for differential measurements was described [4]. Several methods for immobilisation of antibodies onto sensor surface were studied. The stability of self-assembled monolayers prepared with different thiols on gold electrodes in aqueous and organic solvents was studied by capacitive techniques [5]. Short-chain thiols (11-mercaptoundecanoic acid, MUA) displayed a poor stability in aqueous solution in contrast with long-chain thiols (16-mercaptohexanoic acid). Similar methods for the direct detection of antibody–antigen interaction were studied, using immobilisation of antibody based on either the self-assembling properties of functional thiols bearing long alkyl chains or the possibility of a direct coupling of antibody moieties [6, 7]. The spontaneous adsorption of antibody on gold was used for detection of immunological interaction between human mammary tumour-associated glycoprotein and the corresponding monoclonal antibody (GP1D8) [8]. The direct monitoring of small molecules was studied using several techniques including the effect of conformation changes during interaction with

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the corresponding antibodies. Antibody attached to bilayer lipid membrane on the surface of a conductometric sensor was used for detection of 2,4-D [9]. The conformation changes of metallothioneins immobilized on self-assembled thiol layers were used for subnanomolar detection of heavy metals using capacitance measurements [10]. Recently, direct impedimetric affinity sensors based on immobilized oligonucleotide and antibodies on bilayer surface were presented [11].

The impedance system described in this paper involved three types of sensors tested for immunochemical detection of 2,4-D. 2,4-D is a pesticide widely used in agriculture for protection of corn fields against weeds [12]. Several immunotechniques for its assay were described, involving ELISA [13–17], ion-selective field effect transistor [18], potentiometric sensors [19] and piezoelectric sensors [12] as transducers. Amperometric sensors, which employed peroxidase as a label, have been developed in our laboratory [20, 21]. This work studies possibilities of impedance devices for a direct immunospecific detection of small molecules represented by the model hapten 2,4-D.

1.1. Experimental

1.1.1. Chemicals

Cystamine dihydrochloride and human serum albumin (HSA) were obtained from Sigma (St. Louis, USA), 4-aminothiophenol (ATPh), 2,4-dichlorophenoxyacetic acid (2,4-D), *O*-(*N*-hydroxysuccinimidyl)-*N*,*N*,*N*′,*N*′-tetramethyluronium tetrafluoroborate (TSTU) and 3,3′-dithiopropionic acid di-(*N*-succinimidyl ester) (DTSP) were obtained from Fluka (Buchs, Switzerland); glutaraldehyde was from Reanal (Budapest, Hungary). 11-Mercaptoundecanoic acid was obtained from Aldrich; monoclonal antibody (MAb) anti-HSA was provided by Exbio (Prague, Czech Republic) and MAb against 2,4-D was prepared at Veterinary Research Institute (Brno, Czech Republic). All aqueous solutions were prepared from Millipore water and degassed before use.

1.1.2. Preparation of immunosensors

Three types of sensors were used to study antigen–antibody interactions. The screen-printed electrochemical strip sensors with either disc or finger-like electrode structures were supplied by Krejčí Engineering (Tišnov, Czech Republic). The former one consisted of gold-based working and auxiliary electrodes, the reference and contacts were from the silver layers placed on an alumina support (25 × 7 mm); only reference and working electrodes were used for measurements. The geometric area of working electrodes of 10 randomly chosen strip sensors was calculated from digitally captured microscopic images using the software Quantity One (Biorad, Hercules, CA, USA). The finger sensor consisted of two electrode sets (2.5 × 2.5 mm), containing five lines of electrodes (Fig. 1). The interdigitated array electrodes (IDA) were kindly provided by Prof. V. Tvarožek (Slovak Technical University, Bratislava, Slovakia). The IDA electrode consisted of a pair of microband array electrodes (finger thickness 5 μm with spacing 3 μm).

MAb E2/G2 anti-2,4-D was immobilised on the sensor surface using different methods. At the beginning, the sensors were carefully washed in acetone for 10 min. The working electrode was then coated with a drop of thiocompound (90 μmol l^{-1} cystamine in water, 0.16 mmol l^{-1} 4-aminothiophenol in dimethylsulfoxide, DMSO), and allowed to incubate for 2 h at room temperature. The amino groups from these deposited self-assembled monolayers were activated with glutaraldehyde (3% in water, 1-h incubation at room temperature). Alternatively, activation using 11-mercaptoundecanoic acid was performed with the solution of TSTU. A 500-μl sample of 25 mmol l^{-1} MUA solution in DMF was mixed with the same amount of 50 mmol l^{-1} solution of TSTU in DMF. The prepared mixture was stirred for 24 h and used for activation of sensor surface

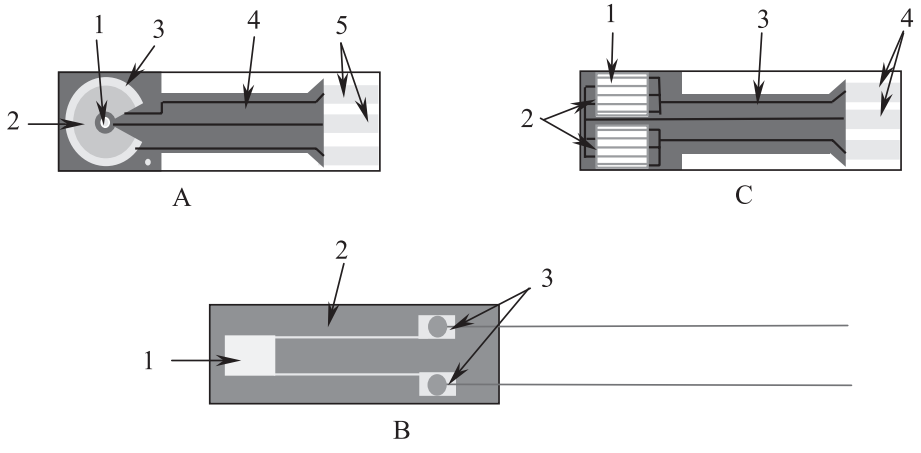

Fig. 1. Schemes of electrodes: (A) Strip sensor: 1 – working electrode, 2 – reference electrode, 3 – auxiliary electrode, 4 – alumina support, 5 – contacts. (B) IDA electrode: 1 – Pt working interdigitated electrode, 2 – connection (interdigitated array contacts), 3 – contacts. (C) Finger strip sensor: 1 – working electrodes, 2 – electrode sets, 3 – alumina support, 4 – contacts.

(24 h). The last tested activation method involved incubation of the sensor surface with solution of DTSP (50 μmol l^{-1}) in DMSO for 2 h. The final step for all three procedures was always the incubation with anti-2,4-D MAb (0.44 mg ml^{-1} in phosphate buffer, 50 mmol l^{-1}, pH 7.0) overnight at 4 °C. Thus, obtained immunosensors with covalently attached anti-2,4-D MAb were stored dry in the refrigerator.

1.1.3. Experimental setup for EIS

The measuring electrodes of the anti-2,4-D Ab modified sensors were immersed in a solution containing PBS (phosphate 50 mM, pH 7, containing sodium chloride, 145 mM), and the response was observed without any stirring at either variable or fixed frequency; the effect of addition of the 2,4-D hapten was evaluated. Small additions of 2,4-D standard solutions prepared in PBS did not influence the ionic concentrations. The measurements were performed at room temperature (25 ± 1 °C). For discontinuous stationary measurements, the time interval for incubation of the immunosensor in the presence of hapten was 30 min.

The sensors were connected to the electrochemical impedance spectrometer EIS 21 (constructed by J. Kitlička, Brno, Czech Republic), the AC amplitude was 5 mV, and the frequency range was from 1 to 10,000 Hz. The output from the detector was transferred to computer using standard serial interface. The program LabTools EIScope under Windows controlled data storage, display and manipulation.

1.1.4. Evaluation of experimental results

The EIS device provided time, frequency, real and imaginary parts of current as results. The rest of parameters (real reZ and imaginary imZ parts of impedance, phase angle ϕ and magnitude $|Z|$) were calculated:

$$\mathrm{reZ} = \frac{U \cdot \mathrm{reI}}{\mathrm{reI}^2 + \mathrm{imI}^2} \tag{1}$$

and

$$-\mathrm{imZ} = \frac{U \cdot \mathrm{imI}}{\mathrm{reI}^2 + \mathrm{imI}^2} \tag{2}$$

$$|Z|^2 = \mathrm{reZ}^2 + \mathrm{imZ}^2 \tag{3}$$

and

$$\phi = \arctan\frac{\mathrm{imZ}}{\mathrm{reZ}} \tag{4}$$

The obtained impedance spectra were analysed using equivalent circuit model. The best fit for biolayer immobilised on the sensor surface was obtained with the circuit containing two resistances and two constant phase elements (CPE) in parallel (Fig. 2) [22], where CPE_1 approximates capacitance of the metal-film interface, R_2 is resistance across the biolayer, and CPE_2 corresponds to capacitance of the biolayer. The program ZView 2 (Scribner Associates, Southern Pines, NC, USA) was used for calculations. The impedance of CPE is given by the equation:

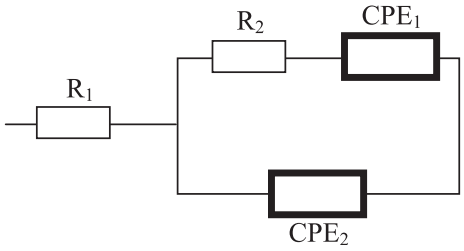

Fig. 2. Equivalent circuit used for evaluation of gold electrodes with immobilized biolayers.

$$Z = \frac{1}{A(j\omega)^{\varphi}} \tag{5}$$

where A indicates constant, ω angular frequency, j imaginary unit, and φ is phase angle (it varies from 0 to 1). When φ is close to 0, the CPE represents resistance; when φ moves between 0.9 and 1, CPE can be considered as capacitance.

The dependence of the relative change of impedance parameters ($Z_{\min}$ and $\phi_{\max}$) on the mole concentration of analyte c was fitted to the sigmoidal equation:

$$n = A_2 + \frac{A_1 - A_2}{1 + (c/c_o)^p} \tag{6}$$

where A_1, A_2, c_o and p are parameters of the fitted equation, c_o indicates the middle point of the assay. The nonlinear regression module from Origin (Microcal, Northampton, USA) was used for all numerical fitting calculations.

2. Results and discussion

2.1. Immobilisation of biorecognition elements

At the beginning, the optimal modification of the sensor surface was studied. Four different coatings of the strip gold electrodes were prepared including cystamine–glutaraldehyde–IgG, aminothiophenol–glutaraldehyde–IgG, DTSP–IgG and MUA–IgG. The result of immobilization of particular components was evaluated using electrochemical impedance spectroscopy. All sensors exhibited the difference in impedance parameters ($\phi_{\max}$,$Z_{\min}$) during the modification steps caused by the surface changes (Table 1). The increasing difference in $\phi_{\max}$ corresponded to the changes of the realignment of bound components on to the sensor surface during immobilisation. On the other hand, the change of impedance Z corresponded to the thickness of the composed biolayer. As evident from the obtained results, for sensors modified with MUA, the biggest increase of impedance was observed probably due to the relatively longest chain provided by MUA. The spectra for the strip sensor modified with aminothiophenol (Nyquist plots) are

Table 1
The changes of impedance minima Z_{min} and capacitances C (CPE_2 according to Fig. 2) within the antibody immobilization procedures for sensors 1–4 based on different self-assembled monolayers

Sensor 1: Au–cystamine–glutaraldehyde–IgG				
	Clean	Cystamine	Glutaraldehyde	Anti-2,4-D
Z_{min} (Ω)	622 ± 31	554 ± 42	590 ± 24	596 ± 20
C (nF)	170 ± 20	169 ± 23	187 ± 21	200 ± 20

Sensor 2: Au–4–aminothiphenol–glutaraldehyde–IgG				
	Clean	ATPh	Glutaraldehyde	Anti-2,4-D
Z_{min} (Ω)	650 ± 15	657 ± 72	643 ± 23	651.6 ± 6.0
C (nF)	169 ± 43	137 ± 18	99.0 ± 6.8	89.7 ± 5.6

Sensor 3: Au–DTSP–IgG			
	Clean	DTSP	Anti-2,4-D
Z_{min} (Ω)	649.0 ± 1.8	610 ± 26	659 ± 16
C (nF)	80.4 ± 5.4	79.7 ± 5.2	86.7 ± 7.1

Sensor 4: Au–MUA–IgG			
	Clean	MUA	Anti-2,4-D
Z_{min} (Ω)	645 ± 14	650 ± 26	678 ± 10
C (nF)	170 ± 26	91.6 ± 5.8	92.8 ± 6.3

Mean value and standard deviation of parameters are shown.

illustrated in Fig. 3. This thiocompound, as was studied before [23], is able to bind on gold surface with a very high density and hence to provide high density of bound antibodies. Therefore, this method of modification was used for the preparation of the following electrodes (IDA, finger strip electrodes).

In addition, the equivalent circuit shown in Fig. 2 was used for characterisation of surface changes during immobilization steps. For all measurements, the value of phase angle φ for CPE_2 moved around the value 0.93 ± 0.03; hence, this constant phase element behaved as a capacitance of the immobilized biolayer. The changes of capacitance observed during different immobilisation procedures are summarized in Table 1. The decrease of capacitance was observed for antibody immobilizations using aminothiophenol and mercaptoundecanoic acid. The same phenomenon was observed in the literature, when the thioctic acid was bound on the electrode surface [24]. On the contrary, the increase of capacitance during immobilisation of thiocompounds cystamine and DTSP indicated the dependency of impedance parameters on the structure of presented biomolecules and character of the formed bonds.

In order to study possible nonspecific interactions, the second set of the screen-printed electrodes was coated with the antibody specific against human serum albumin.

2.2. Monitoring of immunochemical interactions

The produced sensors were tested for the determination of hapten (2,4-D, concentrations from 45 nmol l^{-1} to 450 μmol l^{-1}) using incubation procedure (30-min steps, stationary measurement). The concentration of 45 μmol l^{-1} was also used for continuous measurements (strip sensors only), where the hapten was added to the measuring solution, and binding in real time was followed continuously.

For the stationary measurements, the Bode plot (the dependence of impedance Z on frequency f) exhibited a minimum with amplitude corresponding to concentration of the bound hapten. The best results were obtained for strip sensor with antibody immobilised via DTSP; the lowest response was observed for the sensor based on cystamine monolayer. The IDA electrode provided low reproducibility and sensitivity especially for high concentrations of 2,4-D. The same type of sensor with the same modification provided either decrease or increase of the measured impedance using the same concentrations of 2,4-D.

The nonspecific interactions were evaluated using screen-printed finger strip electrodes. At the beginning, measurements were performed in PBS without any hapten to characterise the sensor stability in solution. Large fluctuations of signal were observed for the electrode set with anti-HAS antibody. 2,4-D was then introduced in concentrations 0, 4.5, 45 and 450 μmol l^{-1}. The interactions of 2,4-D with both anti-2,4-D and anti-HSA immobilised MAbs were compared in Table 2. The changes of Z_{min} and ϕ_{max} are shown in Figs. 4 and 5, respectively. The electrode set with the anti-2,4-D MAb provided sufficient response for the concentration 4.5 μmol l^{-1} of 2,4-D. Nonspecific binding of 2,4-D for the electrode set with the anti-HSA MAb was observed, probably because of the previously noted fluctuations.

Another evaluation method involved characterisation of antigen–antibody complex formation by changes of ϕ_{max} (Bode plot, summarised in Fig. 5). The strip sensors with cystamine and aminothiophenol provided sufficient responses for the low concentration of 2,4-D (4.5 μmol l^{-1}). The strip sensors with DTSP and MUA and IDA electrodes were able to provide response to 2,4-D at rather high concentration (450 μmol l^{-1}). A high resolution was

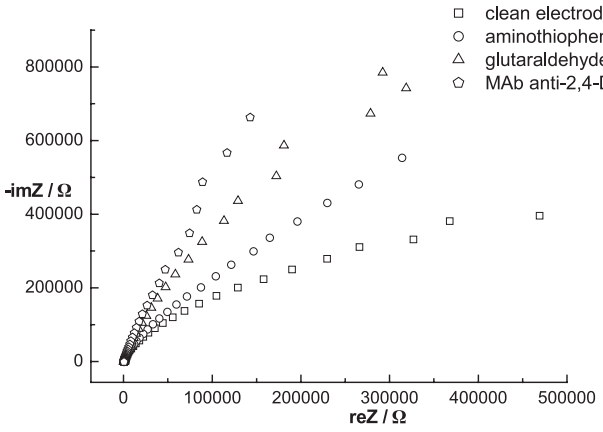

Fig. 3. Nyquist plots obtained during immobilisation procedure for the strip sensor deposition of aminothiophenol–glutaraldehyde–anti-2,4-D antibody layers.

Table 2
Interaction of different types of immunosensors with 2,4-D

(a) Strip sensors

$c_{2,4\text{-D}}$ (μmol l^{-1})	S1 (cystamine)		S2 (ATPh)		S3 (DTSP)		S4 (MUA)	
	ϕ_{max} (°)	Z_{min} (Ω)	ϕ_{max} (°)	Z_{min} (Ω)	ϕ_{max} (°)	Z_{min} (Ω)	ϕ_{max} (°)	
0	611 ± 32	79.1	664 ± 21	81.2	670 ± 44	81.9	705 ± 11	83.0
4.5	612 ± 16	79.9	641 ± 43	81.4	646 ± 33	81.5	667 ± 24	83.3
450	591 ± 36	79.5	609 ± 32	81.3	595 ± 32	82.0	659 ± 21	84.0

(b) IDA electrodes

$C_{2,4\text{-D}}$ (μmol l^{-1})	Z_{min} (Ω)	ϕ_{max} (°)
0	752 ± 42	81.0
4.5	674 ± 36	82.6
450	811 ± 40	83.5

(c) Strip finger electrode

Antibody	anti-2,4-D		Anti-HSA	
$c_{2,4\text{-D}}$ (μmol l^{-1})	Z_{min} (Ω)	ϕ_{max} (°)	Z_{min} (Ω)	ϕ_{max} (°)
0	409 ± 53	76.4	554 ± 19	78.6
4.5	331 ± 40	80.9	333 ± 92	81.3
45	342 ± 43	80.6	518 ± 62	80.5
450	338 ± 41	80.3	519 ± 36	80.3

The values of minimum of impedance Z_{min} (mean values and standard deviations, $n = 3–4$) and phase angle maxima ϕ (mean values, relative standard deviations typically below 5%) for (a) strip sensors with different surface modifications, (b) IDA electrodes, (c) strip finger electrodes observed after addition of 2,4-D.

obtained using finger strip sensor. With respect to specificity of the interaction, the electrode set carrying the MAb anti-2,4-D provided higher response for 2,4-D (4.5 μmol l^{-1}) than the electrode set with anti-HSA.

The change of capacitance during interaction with 2,4-D in increasing concentrations varied for different sensor types (Table 3). Increase of capacitance was observed for strip sensors with aminothiophenol and DTSP and for finger strip sensors. For the strip sensors with cystamine, mercaptoundecanoic acid and IDA electrodes the capacitance decreased. Capacitance of CPE_2 is influenced by the roughness and porosity of electrode surfaces. Hence, for different types of immunosensors, this parameter provides valuable information on the recognition layer.

Continual measurements were carried out at a fixed frequency (2954 Hz) with immersion of the electrode to the vial with 1.8 ml of the measuring buffer. When the baseline achieved its steady state, hapten 2,4-D (0.2 ml) in concentration of 450 μmol l^{-1} was injected to the vessel to get the final concentration 45 μmol l^{-1}. The interaction between antibody and hapten was followed for 8 h. Then, a fresh buffer was introduced, and regeneration of sensing surface was studied after stabilisation of signal. A 0.1-mol l^{-1} sample of HCOOH was used as a regeneration solution.

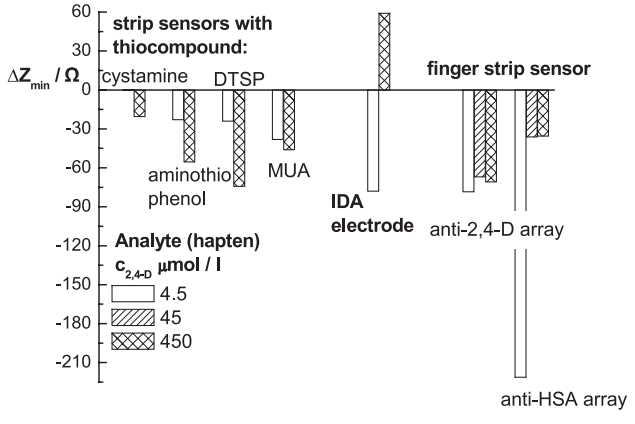

Fig. 4. The changes of the minimum of the impedance Z_{min} in the dependence on the different concentration of 2,4-D (screen-printed strip sensor, IDA electrode, finger strip sensor).

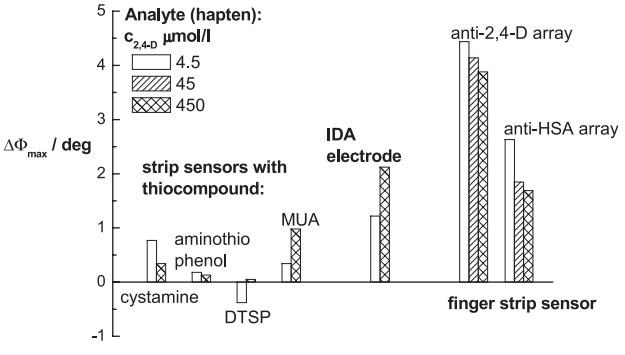

Fig. 5. The changes of the phase angles ϕ_{max} in the dependence on the response for different concentration of 2,4-D (screen-printed strip sensor, IDA electrode, finger strip sensor).

Table 3
The changes of capacitance C for different types of immunosensors obtained in the presence of increasing concentrations of hapten (2,4-D)

(a) Strip sensors

$c_{2,4\text{-D}}$ (μmol l^{-1})	S1 (cystamine)	S2 (ATPh)	S3 (DTSP)	S4 (MUA)
	C (nF)			
0	170 ± 20	87.1 ± 5.7	86.7 ± 7.1	98.5 ± 5.7
4.5	161 ± 18	91.2 ± 6.8	95.2 ± 8.8	97.5 ± 2.1
450	182 ± 19	92.5 ± 5.9	94.5 ± 7.0	90.5 ± 1.8

(b) IDA electrode

$c_{2,4\text{-D}}$ (μmol l^{-1})	C (nF)
0	67.3 ± 2.8
4.5	64.3 ± 2.9
450	59.0 ± 2.0

(c) Strip finger electrode

el. set	Anti-2,4-D	Anti-HSA
$c_{2,4\text{-D}}$ (μmol l^{-1})	C (nF)	
0	395 ± 24	308.1 ± 4.9
4.5	533 ± 29	498 ± 22
45	564 ± 17	514 ± 19
450	565 ± 16	523 ± 20

The obtained responses are shown in Fig. 6. The additions of 2,4-D caused responses 38.2, 38.3, and 40.9 Ω after regeneration. A slow decrease of signal was then observed caused probably by the antibody–hapten complex formation. Following the second repeated addition of 2,4-D, a jump of the signal (77 Ω) was observed, when the working solution was replaced with a fresh buffer. It was probably partially due to defects in connections between sensor and measuring device, when the sensor was removed and reinserted into the liquid medium.

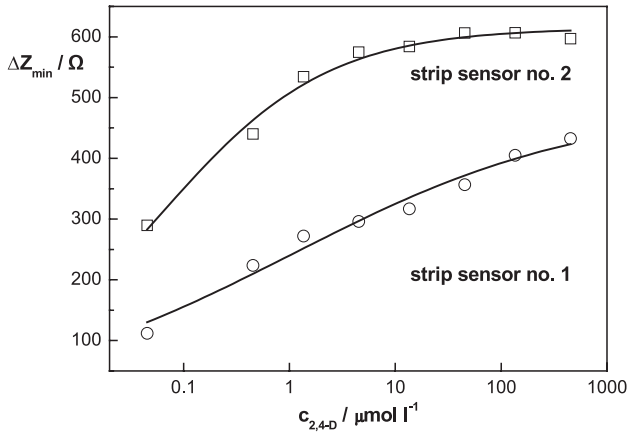

Fig. 7. Calibration curves for two individual strip sensors (Au–aminothiophenol–glutaraldehyde–anti-2,4-D): sensor 1 (○), sensor 2 (□); the dependence of ΔZ_{min} on the increasing concentration of 2,4-D; sigmoidal fit was used for the approximation of the calibration curves.

2.3. Calibration curve for 2,4-D

According to the obtained results, calibration curves for increasing concentration of 2,4-D were constructed. For this purpose, the strip sensor with immobilized antibody anti-2,4-D via aminothiophenol and glutaraldehyde was chosen. In Figs. 7 and 8, the calibration curves for two individual sensors are presented. Apparent difference between shapes of calibration curves indicate that strip sensors used in this work were not entirely the same. In fact, the main difference consisted especially of the size and shape of the gold working electrode surface that was not exactly reproducible. The area of the working electrode ranged from 0.368 to 0.655 mm^2 within 10 individual randomly chosen sensors,

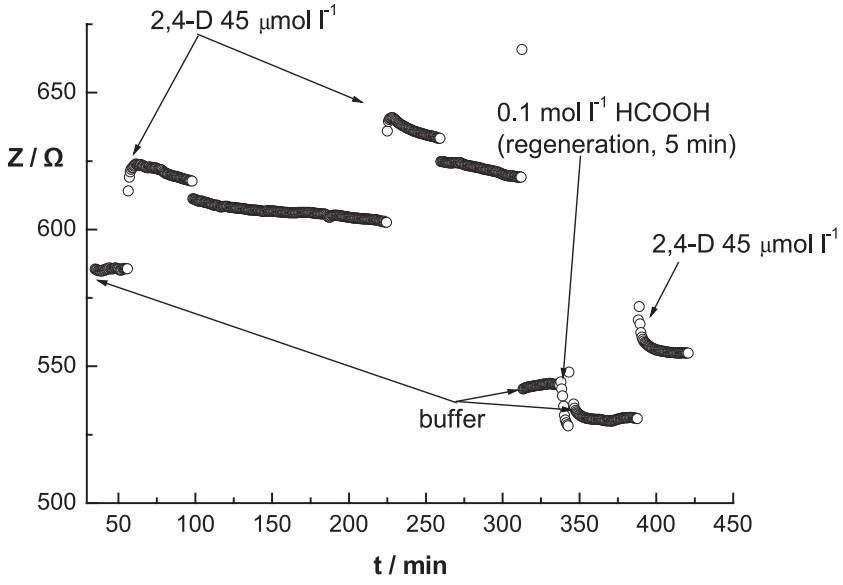

Fig. 6. Continual measurement (sensor Au–cystamine–glutaraldehyde–IgG): injection of 2,4-D (45 μmol l^{-1}), regeneration of sensor surface with 0.1 M HCOOH. Arrows indicate addition of particular components to measuring buffer.

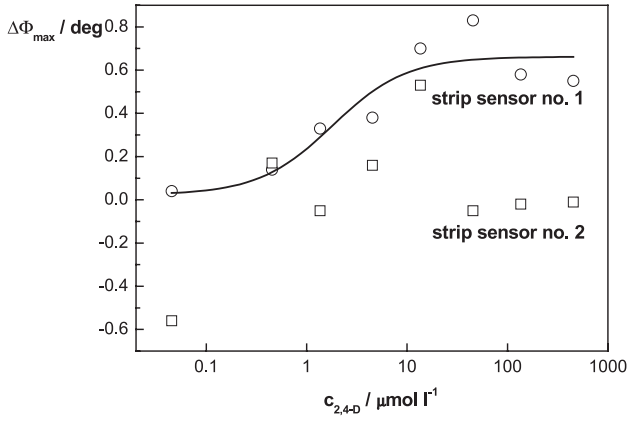

Fig. 8. Calibration curves for two individual strip sensors (Au–aminothiophenol–glutaraldehyde–anti-2,4-D): sensor 1 (○), sensor 2 (□); the dependence of $\Delta\phi_{max}$ on the increasing concentration of 2,4-D; sigmoidal fit was used for the approximation of one of the calibration curves.

mean value was 0.518 ± 0.082 mm^2. Nevertheless, when using only one sensor repeatedly, the calibration curve constructed from the impedance parameters dependency provided reasonable results. For practical evaluation, the calibration curves were approximated with the sigmoidal function (Eq. (5)). Thus, obtained parameters are summarised in Table 3. Using this method, it was possible to measure response to 2,4-D in concentration range from 45 nmol l^{-1} to 450 μmol l^{-1}. Detection ranges for 2,4-D available in the literature vary significantly according to the type of transducer used. The detection limit for 2,4-D previously described direct capacitance immunosensor was around 50 μmol l^{-1} [9]. The immunosensor based on lipid films supported on thin gold layers achieved the detection limit 1 μmol l^{-1} [25]. Competitive amperometric immunosensor developed in our laboratory achieved LOD 0.45 nmol l^{-1} [21]. Direct piezoelectric immunosensor was able to detect 2,4-D in concentration 22.6 nmol l^{-1} and competitive immunosensor 45 pmol l^{-1} [26] (Table 4).

For increasing concentrations of 2,4-D, the calibration curves performed using strip sensors modified with aminothiophenol exhibited a continual decrease of capacitance CPE_2 from 190 to 42 nF (sensor 1) and from 90 to 34 nF (sensor 2) followed by a slight increase of phase angle (Table 5). The decrease of capacitance upon binding of antigen was observed in literature [11, 25]. The different tendencies of both individual strip sensors were probably caused by the low production reproducibility. Nevertheless, it was possible to determine 2,4-D in different concentrations using the same sensor which was successfully regenerated after each measurement.

Table 4
Parameters of the sigmoidal functions approximating the calibration curves (relative response vs. concentration of 2,4-D) for two individual strip sensors

Strip sensor number (evaluated parm.)	A_1	A_2	x_0 (μmol l^{-1})	p
1 (ΔZ_{min})	-3 ± 18	487 ± 53	1.06 ± 0.86	0.31 ± 0.07
2 (ΔZ_{min})	1 ± 15	615 ± 11	0.061 ± 0.011	0.55 ± 0.06
1 ($\Delta\Phi_{max}$)	0.023 ± 0.090	0.660 ± 0.079	1.8 ± 1.2	1.18 ± 0.78

Table 5
The values of capacitances C of biolayers (CPE_2 in Fig. 2) obtained during calibration for 2,4-D performed with two individual immunosensors (screen-printed sensor, antibody immobilized to aminothiophenol using glutaraldehyde)

$c_{2,4\text{-D}}$ (μmol l^{-1})	Sensor 1, C (nF)	Sensor 2, C (nF)
0	190 ± 65	90 ± 11
0.045	104 ± 35	58 ± 12
0.45	64.8 ± 8.3	30.5 ± 3.7
1.36	53.1 ± 6.5	33.8 ± 2.9
4.5	50.9 ± 3.9	32.9 ± 2.7
13.6	48.6 ± 2.9	33.1 ± 2.9
45	35.0 ± 6.7	33.0 ± 2.9
136	43.0 ± 3.2	33.5 ± 3.2
450	42.1 ± 2.4	33.8 ± 3.2

3. Conclusions

EIS in connection with immunochemical methods was tested for the direct determination of the herbicide 2,4-D. Four different methods of antibody immobilisation were studied. Impedance spectra were obtained and evaluated for each step of the modification process. The changes of ϕ_{max} and Z_{min} with frequency served as a parameter characterising changes on the sensing surfaces. Measurements were performed in frequency range from 1 to 10 kHz. The most sensitive responses for Z_{min} were obtained at frequencies exceeding 3 kHz. The best method for covalent immobilisation of antibody employed the self-assembled monolayer of 4-aminothiophenol activated using glutaraldehyde. Three types of sensors were tested (strip sensors, IDA electrodes, and strip finger sensors). Each of them provided similar responses in the presence of hapten 2,4-D depending especially on the sensitivity of each sensor. The real-time experiments showed possibility of regeneration of sensor surface using formic acid. However, this type of measurement was abandoned because of undefined measuring conditions for the subsequent measurements (loss of the contact between sensor and measuring device during rapid changes of medium). Hence, all the measurements were carried out in stationary arrangement. Calibration curves were constructed and evaluated for two individual strip sensors in the concentration range from 45 nM to 0.45 mM of 2,4-D. Using suitable equivalent circuit, it was possible to calculate capacitance changes of the sensor surface occurring during the modification of biolayers. For practical use, the direct use of impedance value obtained at a fixed frequency seems to be satisfactory. The observed direct interactions of immobilised antibody with free molecules of

hapten represented by 2,4-D provided promising base for future experiments.

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References

[1] C. Gabrieli, Physical Electrochemistry, Principles, Methods, and Applications, Department of Materials and Interfaces, Weizmann Institute of Science, Rehovot, Israel, 1995, pp. 243–292.
[2] G. Farace, G. Lillie, T. Hianik, P. Payne, P. Vadgama, Reagentless biosensing using electrochemical impedance spectroscopy, Bioelectrochemistry 55 (2002) 1–3.
[3] O. Ouerghi, A. Touhami, N. Jaffrezic-Renault, C. Martelet, H. Ben Ouada, S. Cosnier, Impedimetric immunosensor using avidin–biotin for antibody immobilization, Bioelectrochemistry 56 (2002) 131–133.
[4] H. Maupas, A.P. Soldatkin, C. Martelet, N. Jaffrezic-Renault, B. Mandrand, Direct immunosensing using differential electrochemical measurements of impedimetric variations, J. Electroanal. Chem. 421 (1997) 165–171.
[5] M. Riepl, V.M. Mirsky, I. Novotný, V. Tvarožek, V. Reháček, O.S. Wolfbeis, Optimization of capacitive affinity sensors: drift suppression and signal amplification, Anal. Chim. Acta 392 (1999) 77–84.
[6] S. Ameur, C. Martelet, N. Jaffrezic-Renault, J.M. Chovelon, Sensitive immunodetection through impedance measurements onto gold functionalized electrodes, Appl. Biochem. Biotechnol. 89 (2000) 161–170.
[7] M. Šnejdárková, L. Csaderová, M. Rehák, T. Hianik, Amperometric immunosensor for direct detection of human IgG, Electroanalysis 12 (2000) 940–945.
[8] M. Jie, C.Y. Ming, D. Jing, L.S. Cheng, L.H. Na, F. Jun, C.Y. Xiang, An electrochemical impedance immunoanalytical method for detecting immunological interaction of human mammary tumor associated glycoprotein and its monoclonal antibody, Electrochem. Commun. 1 (1999) 425–428.
[9] T. Hianik, V.I. Passechnik, M. Šnejdárková, B. Sivak, M. Fajkus, S.A. Ivanov, M. Fránek, Affinity biosensors based on solid supported lipid membranes. Their structure, physical properties and dynamics, Bioelectrochem. Bioenerg. 47 (1998) 47–55.
[10] I. Bontidean, C. Berggren, G. Johansson, E. Csoregi, B. Mattiasson, J.A. Lloyd, K.J. Jakeman, N.L. Brown, Detection of heavy metal ions at femtomolar levels using protein-based biosensors, Anal. Chem. 70 (1998) 4162–4169.
[11] M.Y. Vagin, E.E. Karyakina, T. Hianik, A.A. Karyakin, Electrochemical transducers based on surfactant bilayers for the direct detection of affinity interactions, Biosens. Bioelectron. 18 (2003) 1031–1037.
[12] J. Horáček, P. Skládal, Improved direct piezoelectric biosensor operating in liquid solution for the competitive label-free immunoassay of 2,4-dichlorophenoxyacetic acid, Anal. Chim. Acta 347 (1997) 43–50.
[13] D.F. Rinder, J.R. Fleeker, A radioimmunoassay to screen for 2,4-dichlorophenoxyacetic acid and 2,4,5-trichlorophenoxyacetic acid in surface water, B. Environ. Contam. Tox. 26 (1981) 375–380.
[14] J. Fleeker, Two enzyme immunoassays to screen for 2,4-dichlorophenoxyacetate acid in water, J. Assoc. Off. Anal. Chem. 70 (1987) 874–878.
[15] J.C. Hall, R.J.A. Deschamps, K.K. Kreig, Immunoassays for the detection of 2,4-D and picloram in river, water and urine, J. Agric. Food Chem. 37 (1989) 981–984.
[16] T.S. Lawruk, C.S. Hottenstein, J.R. Fleeker, J.C. Hall, D.P. Herzog, F.M. Rubio, Quantification of 2,4-D and related chlorophenoxy herbicides by a magnetic particle-based ELISA, B. Environ. Contam. Tox. 52 (1994) 538–545.
[17] M. Fránek, V. Kolář, M. Granátová, M. Nevoránková, Monoclonal ELISA for 2,4-Dichlorophenoxyacetic acid: characterization of antibodies and assay optimisation, J. Agric. Food. Chem. 42 (1994) 1369–1374.
[18] S.M. Khomutov, A.V. Zhedrev, B.B. Dzantiev, A.N. Reshetilov, Immunodetection of herbicide 2,4-dichloro-phenoxyacetic acid by field-effect transistor-based biosensors, Anal. Lett. 27 (1994) 2955–2983.
[19] B.B. Dzantiev, A.V. Zherdev, M.F. Yulaev, R.A. Sitdikov, N.M. Dmitrieva, I.Y. Moreva, Electrochemical immunosensors for determination of the pesticides 2,4-dichlorophenoxyacetic and 2,4,5-trichlorophenoxyacetic acids, Biosens. Bioelectron. 11 (1996) 179–185.
[20] T. Kaláb, P. Skládal, A disposable amperometric immunosensor for 2,4-dichlorophenoxyacetic acid, Anal. Chim. Acta 304 (1995) 361–368.
[21] P. Skládal, T. Kaláb, A multichannel immunochemical sensor for determination of 2,4-dichlorophenoxyacetic acid, Anal. Chim. Acta 316 (1995) 73–78.
[22] A.A. Karyakin, M.Y. Vagin, M.A. Choba, N.L. Klyachko, Self-assembled amphiphilic bilayers of surfactant Brij-52 on gold electrodes, Electroanalysis 11 (1999) 1094–1097.
[23] J. Horáček, Diploma thesis: Piezoelectric biosensors, Masaryk University, 1996.
[24] C. Berggren, G. Johansson, Capacitance measurements of antibody–antigen interactions in a flow system, Anal. Chem. 69 (1997) 3651–3657.
[25] T. Hianik, M. Šnejdárková, L. Sokolíková, E. Meszar, R. Kriványek, V. Tvarožek, I. Novotný, J. Wang, Immunosensors based on supported lipid membranes, protein films and liposomes modified by antibodies, Sens. Actuators B 57 (1999) 201–212.
[26] J. Halámek, M. Hepel, P. Skládal, Investigation of highly sensitive piezoelectric immunosensors for 2,4-dichlorophenoxyacetic acid, Biosens. Bioelectron. 16 (2001) 253–260.