INDIVIDUAL AND SIMULTANEOUS DETERMINATION OF SOME PESTICIDES BY BIOSENSORS

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Abstarct

It was elaborated electrochemical (based on ion-sensitive field effect transistors - ISFETs and electrolyte-insulator-semiconductors – EIS-structures) and optical (based on surface palsmon resonance – SPR) immune sensors for the determination of simazine (2-chloro-4,6-di(N-ethylamino)-1,3,5-triazine) and 2,4-D (2,4-dichlorophenoxyacetic acid). The sensitivity of electrochemical sensors was much less at the competitive analysis in comparison with sequential saturation of antibodies, left unbound after their exposure to native herbicide in investigated sample, with labelled herbicide. If horse radish peroxidase (HRP) label was replaced by β-glucose oxidase (GOD) one the sensitivity of analysis by these sensors increased approximately in 5 times. The sensitivity of ISFETs based immune sensor is 0,1 and 0,05 ng/ml for simazine and 2,4-D respectively. The sensitivity of EIS structures and SPR based sensors is less than ISFETs based one. That is why it is recommended to use both first sensors for wide screening of herbicides in environment. The ways for increasing of EIS structures and SPR based immune sensor sensitivity were proposed.

Introduction

Herbicides are mostly often used in agriculture (1). Usually, they are very stable, sufficiently soluble and able to penetrate in the soil as well as to appear in ground- and sea- waters. These substances may induce different human diseases: leucosis, immune deficiency, some mutagenic process and others (1,2). That is why their level in the environment should be at a very strong control. For such control it is necessary to have a very sensitive, selective, simple, fast and cheap analysis. It may be achieved with help of biosensors. In this report we present results obtained by the developed electrochemical immune sensors based on the ISFETs, EIS and optical ones based on SPR. These biosensors are intended for control of simazine and 2,4-D as most widely used compounds in agriculture of different countries.

Methods

The synthesis of simazine- conjugates and obtaining of antiserum to simazine and 2,4-D were described in (3). Conjugates of 2,4-D with proteins and enzymes were obtained in different ways. For conjugation of 2,4-D with bovine serum albumine (BSA) or ovalbumin (Ova) trough carboxyl group it was prepared 10 ml of 0,003 mM of water solution of protein and 1 ml of 0,1 mM of 2,4-D solution in dimethylformamid. Both solutions were mixed and water solved carbodiimide at the final concentration of 0,24 mM was added to the mixture. After that the mixture was stirred up during 8 hours at the room temperature and than it was kept in refrigerator during 12 hours. At the end the mixture was dialyzed against of distilled water, centrifuged and lyophilic dried. For the conjugation of 2,4-D with BSA or Ova trough benzene body it was prepared the solution of 2,4-D in 2 ml of distilled water with the adding of 1M NaOH (to achieve pH 9,0). Then, 1 ml of hydrogen peroxide (50%) and 8 mg of $FeSO_4$ were added to solution above metioned. In parallel 0,005 solution of BSA in 8 ml of distilled water was prepared. Both solutions were mixed and reaction mixture was stirred during 20 mn at 4°C. At last it was dialised against distilled water, centrifugated and lyophilic dried.

Rabbits were immunised two times by subcutaneous injection of emulsion of 2,4-D – BSA (or Ova) conjugate with full Freind adjuvant in ration 1:1. Than rabbits were immunised four times by intramuscular injection without Freind adjuvant. The blood was taken on 7-8 day after last procedure of immunisation. As it was mentioned above two types conjugates were obtained, namely: conjugate I and II. The first of them was prepared in case of the conjugation of appropriate protein with 2,4-D trough carboxyl group and second one – trough body of benzene (Fig. 1). According to these conjugates two types antiserum were obtained too.

In experiments we used antiserum or IgG fraction of specific antibodies, which was prepared by ammonium sulphate precipitation of antiserum. In preliminary experiments it was shown that the titre of antiserum obtained after immunization of rabbits by conjugate II was much higher then in case of use of conjugate I (as a rule at the end of procedure of immunisation the titre was 1:12800 in comparison with 1:6400). Maybe, the conjugation trough body of benzene allows to us to expose this herbicide to immunocompetent elements as whole molecule including carboxyl group and it gives possibility to recognise this hapten more effectively then in case of its conjugation trough carboxyl group.

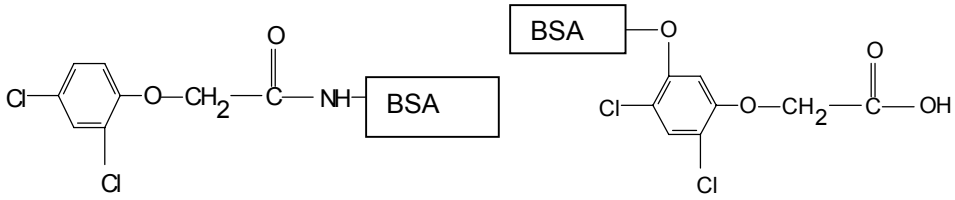


Fig. 1. Two different way for conjugation 2,4-D with BSA.

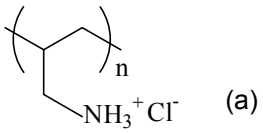
The antiserum to simazine cross-reacted with atrazine (89%), terbutylazine (80%), and propazine (10%). Other analytes demonstrated cross-reaction in the range 0.7-6.2%. Antiserum to 2,4-D had not cross-reaction with simazine. The principals of design and work of electrochemical biosensors were presented in (4-6). Specific antibodies to herbicides were immobilised through the staphylococcal protein A. Two methods of herbicide detection were used in case of ISFET based immune sensor – (i) competitive assay when native (detected) and HRP or GOD -labeled herbicide molecules competed for binding with antibodies on the transducer surface, and (ii) sequential saturation of antibodies, left unbound after their exposure to native herbicide in investigated sample, with labeled herbicide. The last analysis was carried out too with the immune sensor based on the EIS structures. The peculiarity of optical immune sensor design was described in (7) and the main of algorithm of its work indicated in the text and the legends for the presented figures. At the creation of optical immune sensor based on the SPR we used competitive analysis when free and conjugated 2,4-D compete for sites of specific antibodies immobilized on the transducer surface. Specific antibodies were immobilised with the use of intermediate layers: polyelectrolites (Fig. 2) or polyelectrolites with lectins, or polyelectrolites with protein A from *Staphylococcus aureus*.

Results

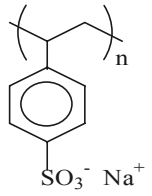
The traditional ELISA-method was curried out by competitive way in two different regimes. In one of them was the following. Herbicide (2,4-D) conjugated with one of proteins above indicated was immobilised on the solid phase and competed with free 2,4-D for binding centres of specific antibodies. The second regime of analysis has foreseen the competition of free and conjugated 2,4-D with HRP for the binding centres of specific antibodies, which was preliminary immobilised on the solid phase. In this experiments we tried to elucidate: 1) what type of conjugates of 2,4-D with proteins (I or II) are better for the analysis; 2) what ratio of 2,4-D and HRP is better at the preparation of conjugates of this herbicide with enzymes to have very high level of immune analysis and c) what from above mentioned regimes can provide more sensitive analysis?

In result of investigation it was established that in case of realisation of both regimes of analysis the best results (according to sensitivity and linear plot of 2,4-D determination) were obtained with conjugates of type II. The best results were too obtained in case of the use of conjugates of 2,4-D with HRP in ratio of 4:1. The maintenance of these conditions at the optimisation of algorithm of analysis allows to us to obtain the sensitivity of analysis at the level of up to 0.1 ng/m. Both examined regimes of analysis given the same sensitivity of 2,4-D determination.

Fig. 2. Polyelecrolites: a - polyalylamine hydrochloride and b - polystyrenesulfonate sodium salt (the details of the immobilization – see in (8).



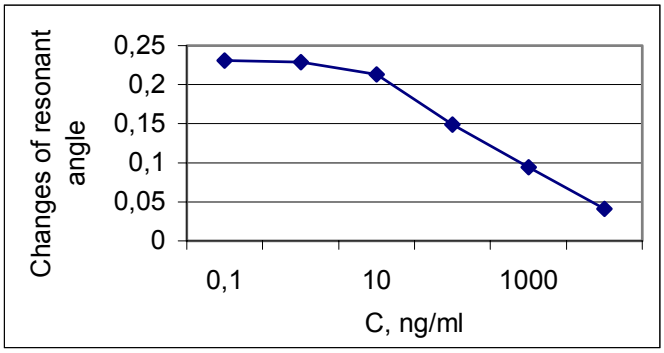
(a)



(b)

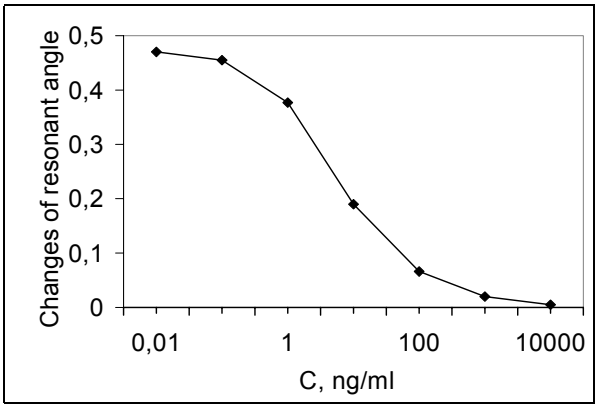
It was shown that in both cases of the immobilisation of specific antibodies from antiserum on the gold surface of SPR (covered by polyelectrolites or polyelectrolites with lectins) we obtained the same sensitivity of 2,4-D determination, namely: about 1 ng/ml (Fig. 3).

Fig. 3. SPR immune sensor response in the presence of different concentrations of 2,4-D in solution. Conditions: specific antibodies (antiserum) are immobilized on the gold surface covered by polyelectrolites. Free and Ova conjugated 2,4-D compete for antibodies.



If we used procedure of immobilization of specific antibodies from antiserum on the gold surface covered by polyelectrolites with protein A from *Staphylococcus aureus* the sensitivity of the 2,4-D determination was increased approximately on one order (Fig. 4).

Fig. 4. SPR immune sensor response in the presence of different concentrations of 2,4-D in solution. Conditions: specific antibodies (antiserum) are immobilized on the gold surface covered by polyelectrolites and protein A. Free and Ova conjugated 2,4-D compete for antibodies.



Nevertheless at the analysis of 2,4-D in corn (model experiments) we can determine the concentration of this herbicide on the level of 0,1 ng/ml (Fig. 5). The model corn composites were prepared by two ways. 1). 2,4-D in different quantities (mass ratio) was added to corn and carefully mixed. Then corn was washed by the solution of ethanol. 2). Corn was reduced to fragments and extracted by tris-HCl buffer with pH of 7,5. Then 2,4-D was added to extract in different concentrations.

At the optimisation of ISFET based immune sensor analysis the limit of simazine detection by competitive assay was 0,65 ng/ml. In case of sequential saturation assay it may be registered 0,1 ng/ml. At 2,4-D revealing by use so called saturation immunoassay we obtained the sensitivity about 0.05 ng/ml and linear plot was in the range of 0.1-130 ng/ml. With the help of competitive analysis it was possible to register 0,1 ng/ml. We compared the results obtained by the immune sensors based on ISFETs and EIS-structures (Table). It was found that the sensitivity of the immune sensor based on EIS-structures was much less (above one order) than that one based on ISFETs.

So, the level of sensitivity of simazine analysis with help of EIS structures and HRP-conjugates was about 5 µg/L. At the some time the linear plot of this sensor response lies in range of concentrations from 5 to 150 µg/L. In these conditions the sensitivity of 2,4-D analysis was 1,25 µg/L and the linear

plot - between 2,5 and 150 μg/L. Of course, this sensitivity of detection for both herbicides was less than it is needed for the practical demands. We tried to elucidate the main reasons of such situation. One of them is connected with the problem of sensor output registration due to formation of air bulbs. The last are as result of intensive HRP activity. Next reason is connected with the specific conditions of this enzyme determination, in particular, with the use of a big concentration of ascorbic acid. This factor stipulates for elimination of charged ions formed at the oxidization of ascorbic acid due to enzymatic reaction. We changed HRP label on the GOD one and obtained the analysis sensitivity approximately in 5 times more than in first one. The linear plots of registered concentrations were 1,0-150,0 and 0,25-150,0 ng/mL for simazine and 2,4-D, respectively.

Fig. 5. SPR immune sensor response in the presence of different concentrations of 2,4-D in solution to be analyzed. Conditions: specific antibodies (antiserum) are immobilized on the gold surface covered by polyelectrolites and protein A. Free (elution from corn) and 2,4-D conjugated with Ova compete for antibodies.

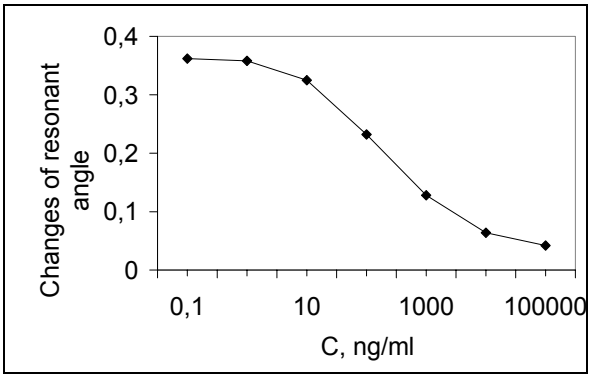


Table. Comparison of the sensitivity of ISFET and EIS-structures based immune sensor to some herbicides.

Herbicides	Level of sensitivity (μg/L) of immune sensor based on:			
	ISFET		EIS	
	type of analysis		type of labels	
	competitive	sequential saturation	HRP	GOD
2,4-D	0,1	0,05	1,25	0,25
Simazine	0,65	0,1	5,0	1,0

Discussion

In spite of non-higher sensitivity of EIS structure based immune sensor it attracts a very big attention in investigators according to the next reasons. One of them is the simplicity of the procedure of analysis and the possibility to fulfil multi-parametrical control of environment and to carry out a lot of repeated tests. For providing of repeated analysis the replaceable membranes are very suitable. The overall time of analysis for both electrochemical immune sensors is the same (about 40 min). There is possible to determine all herbicides simultaneously. The sensitivity and selectivity of analysis may be sharply increased by use monoclonal antibodies and by carrying out of multi-parametrical analysis. The second reason, which excites interest in investigators to the EIS-structures based immune sensor, is the simplicity of its design in the comparison with the ISFETs based one. In addition to the last immune sensor is more expensive since it connects with more precise and complicate technology of transducer creation. At last, third, reason is that the EIS-structures based immune sensor may be used for wide screening of herbicide presence in environment. It gives possibility to curry out analysis of 8-10 samples simultaneously. It may be provide for wide screening not herbicides only and other types of toxicants. At the same time, the ISFETs based immune sensor may serve for verification and conformation of the results of preliminary screening. We believe that the sensitivity of EIS structures based immune sensor could be yet increased. One of the perspective ways to do it is the

development of special suitable membranes. There is necessary to provide a very high density of the immobilised specific antibodies on the membrane surface. Moreover, it would be very effective if these antibodies were immobilized not only on the membrane surface and in its large-scale pores, which would be accessible for big molecules of conjugates herbicides with enzymes. As material for membrane creation we foresee synthetic polymers, which are biological compatible, allow to change density and porosity of membrane in wide range and which are very simple in preparation [9, 10]. Of course, to increase the sensitivity of analysis it would be also very effective to use monoclonal antibodies with high level of affinity to analytes, to choose enzymes as labels with a big level of activity turnover and to provide the preservation of enzyme activity in time of conjugate preparation. At the preliminary preparation of chips (ISFETs) and membranes the time of analysis may be shortened up to 10 min. The preparation of membrane is much simple than chip creation. The membranes are very cheap and their set may preserve a long time at the drying and staying in refrigerator.

As for optical immune sensor based on SPR there is necessary to mention its simplicity. For analysis fulfilment there is not necessary to use any components labelled by enzymes. Unfortunately its sensitivity is nearly to that which was revealed in electrochemical immune sensor based on the EIS-structures. The same as in case the last immune sensor there is number possibilities to improve sensitivity of optical immune sensor, for example, by optimisation of procedure immobilisation of specific antibodies on the transducer surface and by choosing more effective algorithm of analysis. Maybe, replacement of competitive analysis on above indicated one connected with “sequential saturation of antibodies” would be more effective. The further our investigations will be devoted to examination of these possibilities. In any case the developed optical immune sensor is very perspective for wide screening of not pesticides only and other low weight toxic substances in objects of environment.

Acknowledgments

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