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Ion-Sensitive Field Effect Transistor-Based Multienzyme Sensor for Alternative Detection of Mercury Ions, Cyanide, and Pesticide

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Abstract Various groups of industrial and agricultural pollutants (heavy metal ions, cyanides, and pesticides) can be detected by enzymes. Since heavy metal ions inhibit urease, cyanides inhibit peroxidase, organophosphorus and carbamate pesticides inhibit butyrylcholinesterase, these enzymes were co-immobilized into a bovine serum albumin gel on the surface of an ion-sensitive field effect transistor to create a bioprobe that is sensitive to the compounds mentioned above. The sensitivity of the present sensor towards KCN corresponded to 1 μM with 1 min of incubation time. The detection limits for Hg(II) ions and the pesticide carbofuran were 0.1 and 0.5 μM, respectively, when a 10 min sensor incubation time in contaminated samples was chosen. The total time for determining the concentrations of all species mentioned did not exceed 20 min.

Key words: Multi-biosensor, inhibition, mercury ions, cyanide, pesticide

Scientific interest in species that constitute environmental risks inevitably requires analytical devices or systems for determining a wide range of hazardous pollutants. In this respect, biosensors seem to be very promising, as the related devices are generally simple and rapid in response.

Although a lot of work has been done on the development of such bioprobes, almost all of them aim to determine one narrow class of pollutants, like heavy metal ions [6, 8, 10, 13, 17], cyanides [1, 12, 14], and organophosphorus or carbamate pesticides [2, 5, 9, 11, 15]. To improve the versatility of analysis, Cowell *et al.* [4] proposed a model for a multi-sensor that can determine several different pollutants. They used an array of six enzymes to develop a computer model from which the resultant pattern of an inhibition could be interpreted using an artificial neural net. However, this model is rather complex and inappropriate as a portable device for environmental screening. A simple and reliable approach for rapid environmental screening may be co-immobilization of several enzymes into the same biomatrix and investigation of their activities before and after exposure to potentially noxious species.

Accordingly, the current study describes an alternative method for detecting hazardous environmental pollutants such as mercury ions, cyanide, and pesticide. Three enzymes, urease, peroxidase, and butyrylcholinesterase, which are specifically inhibited by each compound, respectively, were individually or simultaneously immobilized to the sensing parts of an ion-sensitive field effect transistor (ISFET), and the sensor response to each compound was compared.

Materials and Methods

Reagents

The urease (EC 3.5.1.5., 12 U/mg) was obtained from Biolar (Lithuania). The horseradish peroxidase (EC 1.11.1.7, 65 U/mg) and butyrylcholinesterase (EC 3.1.1.8., 15 U/mg) from horse serum were purchased from Sigma (U.S.A.). The ISFET structures were obtained from Emocon (Ukraine). All reagents were of pure analytical grade.

Enzyme Immobilization

To prepare the biomembrane for one-pollutant determination, one drop of a solution (about 0.1 μl) containing 5% of each enzyme, 5% bovine serum albumin (BSA), and 10% glycerol in a 10 mM Tris buffer (pH 7.5) was deposited onto the sensitive area of an ISFET. After this procedure, the sensor was placed into saturated glutaraldehyde vapor for 30 min at room temperature, and then dried in air for 15 min.

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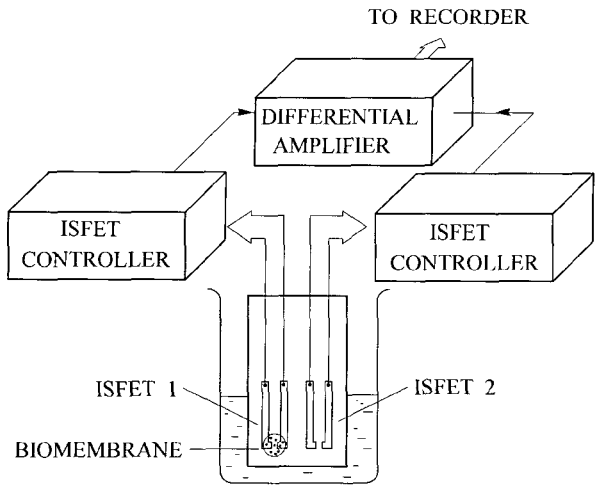

Fig. 1. Experimental set-up.

To prepare the three-enzyme bioprobe, enzyme solutions containing 5% of individual enzyme, 5% BSA, and 10% glycerol in the same buffer, were mixed in equal proportions, and then one drop of the resulting mixture was deposited onto an ISFET. The cross-linking procedure for the enzymes was the same as for the one-enzyme probe.

Sensor Design and Measurements

The sensor chip (dimensions 3 mm×10 mm) had two identical ISFETs. One of them was covered by the biomembrane, while the other served as a reference (Fig. 1). Home-made electronic equipment was used to measure the enzymatically caused pH variations in the biomembrane. The differential signal from the working and reference ISFET was amplified and recorded.

The measurements were carried out at room temperature in a glass cell (1.5 ml) filled with 5 mM Tris buffer (pH 7.5) with 100 mM NaCl as the reaction buffer. To measure the peroxidase-based probe, the buffer solution also contained 15 mM L-ascorbic acid, which was necessary for H_2O_2 formation. The buffer and sample solutions were vigorously stirred during the measurements.

Results and Discussion

Mercuric Ion Detection with Urease-Based Inhibition Sensor

The enzymatically catalyzed hydrolysis of urea caused a pH change in the biomembrane, which was registered in the ISFET (Fig. 1). When the sensor was immersed into a solution containing heavy metal ions, these species inhibited the enzyme, resulting in a reduction of the response signal.

First, the sensor's response to the addition of 20 mM urea was recorded (Fig. 2). Then, the sensor was immersed into the reaction buffer containing a known concentration

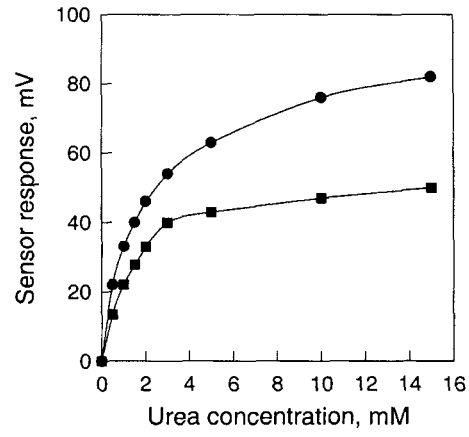

Fig. 2. Responses of one-enzyme (●) and three-enzymes (■) probes to urea addition.

of $HgCl_2$ for 10 min to inhibit the enzyme activity. After rinsing with the buffer solution, the sensor's response to the substrate addition was recorded again. The difference in the signals before and after inhibition was proportional to the inhibitor concentration (Fig. 3).

It has been reported by Preininger and Wolfbeis [10] that heavy metal ions inhibit urease in the following order: Ag(I)>Hg(II)>Cu(II). Thus, it was important to improve the sensor selectivity towards the mercury ions. Therefore, to suppress the enzyme sensitivity to silver ions, a specific anion was added to form an insoluble or slightly soluble salt with silver cations, yet not to influence the inhibition properties of mercury ions. The most effective additional anion seemed to be an iodide anion. Even a slight addition of NaI into the sample solution significantly decreased the sensor's response towards silver ions. It was found that

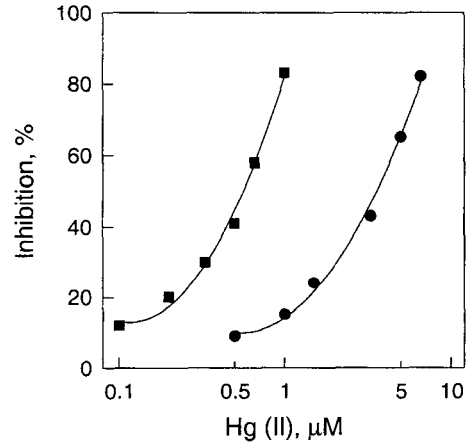

Fig. 3. Sensitivities of one-enzyme (●) and three-enzymes (■) probes to mercury ions.
The percentage of inhibition was calculated as $100\% \times (V - V_i)/V$, where V is the sensor's response to the substrate addition before inhibition and V_i is the sensor's response after inhibition. V and V_i were measured in duplicate and then averaged.

100 μM concentration of NaI in the sample solution protected urease from Ag inhibition up to 95%. However, with the addition of NaI, it was impossible to effectively protect the enzyme from cupric ion inhibition. The current study, however, showed that when the enzyme was inhibited by Cu(II) ions, its activity was restored after 5 min of rewashing in 100 mM EDTA solution. In contrast, EDTA did not restore the urease activity inhibited by Hg(II) ions. Therefore, the selective rewashing made the biosensor nonsensitive to cupric ions.

The enzyme activity inhibited by Hg returned up to 95–100% of the original activity by 5 min rewashing in 300 mM NaI solution. Nonetheless, the sensitivity towards the inhibitor was not sufficiently reproducible for reuse of the same probe many times.

Cyanide Detection with Peroxidase-Based Inhibition Sensor

Owing to the extensive usage of cyanide compounds in the industrial (extraction of gold and silver from ores) and agricultural (fumigation of fruit trees) sectors, the development of cyanide sensors has been closely connected with the topic of environmental pollutant determination. Since some iron-containing enzymes, such as peroxidases, are inhibited by cyanides [16], such bioelements can be used to prepare biosensors for precise cyanide detection.

L-Ascorbic acid is one possible substrate for peroxidase, and its consumption in the biomembrane during an enzymatically catalyzed H_2O_2 reduction caused a local basic pH shift that was registered in the ISFET (Fig. 4). At first, the sensor's response was recorded with the addition of up to 5 mM H_2O_2. Then, the sensor was immersed into the buffer solution containing a known concentration of KCN. After 1 min of incubation, the sensor response to L-ascorbic acid was recorded again. The difference in the signals before and after the inhibition was proportional to

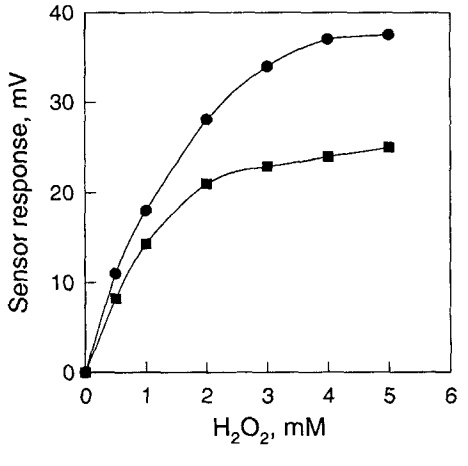

Fig. 4. Responses of one-enzyme (●) and three-enzymes (■) probes to hydrogen peroxide addition.

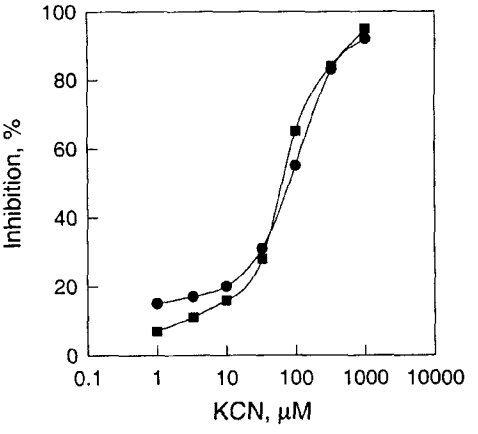

Fig. 5. Sensitivities of one-enzyme (●) and three-enzymes (■) probes to KCN.

The percentage of inhibition was calculated as $100\% \times (V - V_i)/V$, where V is the sensor's response to the substrate addition before inhibition and V_i is the sensor's response after inhibition. V and V_i were measured in duplicate and then averaged.

the inhibitor concentration, and a calibration graph for the determination of cyanide is shown in Fig. 5.

It has been previously reported by Whitaker [16] that azide ions can also suppress the activities of iron-containing enzymes, such as peroxidase, catalase, and cytochrome oxidase. Hence, it was considered important to study the influence of this toxicant on the sensor performance; however, even high concentration of sodium azide (10 mM) was found not to suppress the peroxidase activity.

Because of the reversible nature of peroxidase inhibition by cyanide ions, the enzyme activity after cyanide inhibition was restored by rewashing the sensor in a fresh buffer solution.

Pesticide Detection with Cholinesterase-Based Inhibition Sensor

The high acute toxicity of organophosphorus and carbamate pesticides, which are widely used in agriculture, has generated a need for the development of fast-responding detectors to control food and agricultural samples. As such, the ability of these chemicals to inhibit cholinesterase can be applied for their detection. Acetylcholinesterase and butyrylcholinesterase are usually used for the construction of a bioprobe in which the decrease of enzyme activity in the presence of an inhibitor is monitored.

Since acetylcholinesterase has been reported to be less stable and needs specific reagents, such as Triton X-100, to maintain its activity [5], the immobilization of butyrylcholinesterase on the surface of the ISFET has been proposed in order to construct a pesticide-sensitive bioprobe. Also, Scladal and Mascini [11] demonstrated that this enzyme is more sensitive to certain widely used pesticides, such as paraoxon and heptenophos, than acetylcholinesterase.

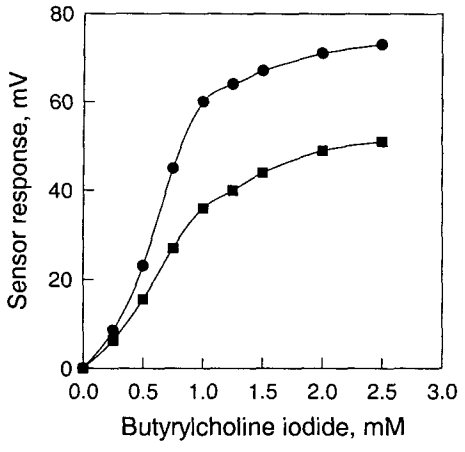

Fig. 6. Responses of one-enzyme (●) and three-enzymes (■) probes to butyrylcholine iodide addition.

The response of the bioprobe to butyrylcholine iodide (Fig. 6) allowed the optimal substrate concentration to be selected for measuring the changes in the enzyme activity caused by an inhibitor. Actually, an injected substrate concentration of 3 mM slightly exceeded the amount of butyrylcholine iodide that corresponded to the beginning of the saturation region in the standard curve.

To study the sensor's sensitivity to a possible cholinesterase inhibitor, the influence of a carbamate pesticide carbofuran on the enzyme activity was examined. First, the sensor response to 3 mM butyrylcholine iodide in 5 mM Tris buffer (pH 7.5) with 100 mM NaCl was recorded. After rinsing with a fresh buffer solution, the probe was immersed into the model pesticide solution for 10 min. Then, the sensor response to the substrate was measured again. The difference in the signals before and after the inhibition was proportional to the pesticide concentration (Fig. 7).

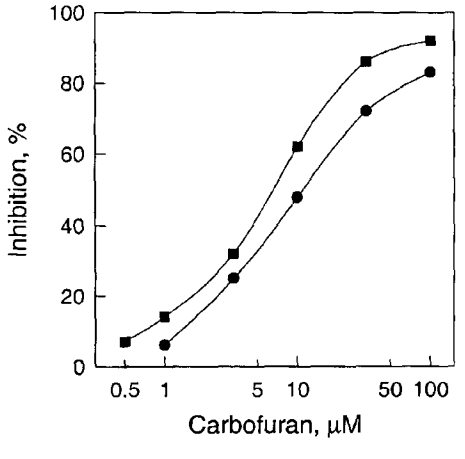

Fig. 7. Sensitivities of one-enzyme (●) and three-enzymes (■) probes to pesticide carbofuran.
The percentage of inhibition was calculated as $100\% \times (V - V_i)/V$, where V is the sensor's response to the substrate addition before inhibition and V_i is the sensor's response after inhibition. V and V_i were measured in duplicate and then averaged.

It has been previously shown by Fennouh *et al.* [5] that the presence of some organic solvents (0.05% acetone, for example) in a buffer solution can considerably increase the sensitivity of a soluble acetylcholinesterase to pesticide paraoxon. Nonetheless, in the current study, even addition of high acetone concentration of 1% into the buffer solution did not influence the sensor's sensitivity to carbofuran, which has the similar inhibition mechanism as paraoxon [5]. The possible reason for this was that, in the presence of an organic solvent, the conformation of the free enzyme was changed, and its active site became more available to the inhibitor. Yet, the conformation of the cross-linked enzyme could not be changed as easily as the free one, thus the access of inhibitor to the active center was not increased.

To restore the enzyme activity inhibited by carbofuran, rewashing the biosensor in a fresh buffer solution for 30–50 min was sufficient. Yet, in case of organophosphorus pesticides, which cause irreversible cholinesterase inhibition, special reactivators are required, such as 2-pyridinealdoxime methiodide (2-PAM) [9, 15] or trimedoxime bromide-4 (TMB-4) [2].

Performance of Three-Enzyme Multi-Biosensor

To form a biomembrane that could be sensitive to the pollutants described above (mercury ions, cyanide, and pesticide), three different solutions containing enzymes that are sensitive to these toxicants were mixed in an equal proportion. Thus, in the case of the multienzyme membrane, the loading of every enzyme was about 17% of the membrane dry weight, which was considerably lower than the 50% content in the case of the one-enzyme biomatrix, thereby potentially reducing the probe's sensitivity to the substrates (Figs. 2, 4, and 6). However, as previously reported by Scladal and Mascini [11], and also shown in Figs. 3, 5, and 7, the decrease in the enzyme loading led to an increase in the probe's sensitivity to the inhibitors. Thus, the multi-biosensor was expected to detect even lower pollutant concentrations than the single-enzyme probe.

The chosen enzymes not only exhibited high responses to the corresponding substrates and inhibitors, but also produced a good inhibitor specificity. That is, there was no sensitivity towards other inhibitors (*e.g.*, cyanide and pesticide in the case of a mercuric ion measurement), when tested with concentrations of up to 1 mM. As shown in Figs. 3, 5, and 7, 1 mM concentration of the inhibitors conspicuously exceeded the dynamic range of the multi-biosensor. Hence, it was concluded that the multi-biosensor in the current study specifically responded to each inhibitor within its operational concentration range.

As the biosensor detection limit for Hg(II) ions was about 0.1 μM (0.02 mg/l), the probe could be used for

mercury detection in fishery products, where the tolerated concentration has been cited as 0.5 ppm (U.S.A. and Canada) or even 1.0 ppm (Sweden, Denmark, and Japan) [3]. The detection limit for cyanide with this bioprobe was about 1 μM (0.027 mg/l), and an ADI value of 50 μg/kg b.w. for cyanide has been reported for rats [3]. For carbofuran, the detection limit was 0.5 μM (0.1 mg/l), whereas a human ADI of 0–0.01 mg/kg has been reported [7].

CONCLUSION

It was demonstrated that an ISFET-based biosensor with a multienzyme membrane can be used for the environmental monitoring of several hazardous pollutants, as it includes the versatile enzyme activities. The sensor was able to separately determine Hg(II) ions, cyanide, and the carbamate pesticide, carbofuran, in water samples that differed in the predominant pollutant species. In addition, the sensor could also be applied to assess the total effect of these chemicals in water samples with all these compounds. Since the co-immobilized enzymes acted independently and did not influence each other, their number could be increased to detect even more pollutants with the same bioprobe.

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