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Feasibility of application of conductometric biosensor based on acetylcholinesterase for the inhibitory analysis of toxic compounds of different nature

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HIGHLIGHTS

- The conductometric biosensor based on acetylcholinesterase was characterized.
- It was applied for inhibitory analysis of different toxins and toxicants.
- Pesticides, heavy metals, surfactants, aflatoxin and glycoalkaloids were determined.
- Studies showed that the different classes of inhibitors can be distinguished.
- Algorithm of analysis of complex multicomponent samples is proposed.

GRAPHICAL ABSTRACT

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ABSTRACT

This study was aimed at the development of a conductometric biosensor based on acetylcholinesterase considering the feasibility of its application for the inhibitory analysis of various toxicants. In this paper, the optimum conditions for enzyme immobilization on the transducer surface are selected as well as the optimum concentration of substrate for inhibitory analysis. Sensitivity of the developed biosensor to different classes of toxic compounds (organophosphorus pesticides, heavy metal ions, surfactants, aflatoxin, glycoalkaloids) was tested. It is shown that the developed biosensor can be successfully used for the analysis of pesticides and mycotoxins, as well as for determination of total toxicity of the samples. A new method of biosensor analysis of toxic substances of different classes in complex multicomponent aqueous samples is proposed.

1. Introduction

In the recent years, environmental monitoring is becoming an increasingly urgent task. This is due to the rapid development of

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chemical industry and intensive use of various chemicals in agriculture and other fields of human activity. One of the promising trends in research for environmental monitoring is biosensorics, namely the development of enzyme electrochemical biosensors for the determination of different classes of toxicants [1]. A significant interest manifested today to biosensors is due to their specific advantages compared to traditional methods of analysis, i.e., relative cheapness and ease-of-use, high sensitivity and specificity.

To determine the toxic substances by biosensors, an inhibitory analysis based on a variety of enzymes (cholinesterase, urease, glucose oxidase, etc.) is commonly applied. Today acetylcholinesterase (AChE) is the most frequently used enzyme, due to its sensitivity to a number of toxic substances–pesticides, glycoalkaloids, etc. [2].

The first biosensor based on the cholinesterases inhibition was developed in 1962 for determination of agents with neuroparalytic action [3]. Since then, a large number of AChE-based biosensors were developed for determination of various toxic substances. Most of them are designed for inhibitory analysis of organophosphorus pesticides [4], others are reported for identification of heavy metals in model [5,6] and real [7] samples, neurotoxins [8], chemical agents of neuroparalytic action [9,10], drugs [11], etc.

Therefore, a conclusion can be made that AChE is inhibited with very wide range of toxic substances. On one hand, it is an advantage of AChE-based biosensors since it allows their application for analyzing numerous substances. On the other hand, the problems occur for analysis of mixture of toxic substances, i.e., when there is a need to distinguish the impact of various toxicants on AChE. Thus, it is reasonable to analyze the total impact of different toxic substances on the AChE-based biosensor.

This work is devoted to the development and investigation of the AChE-based conductometric biosensor for inhibitory analysis of various toxicants (organophosphorus pesticides, heavy metal ions, surfactants, aflatoxins and glycoalkaloids). The aim of this study was to analyze the different types of inhibition of a bioselective element (reversible and irreversible), to consider the feasibility of application of the AChE-based biosensor for the identification of both particular class of toxicants and complex multicomponent mixtures.

2. Methods of research

2.1. Materials

Bioselective membranes contained enzyme acetylcholinesterase (AChE) from electric eel (EC 3.1.1.7), activity 426 U/mg, bovine serum albumin (BSA) (fraction V, purity ≥ 98.0%), 50% aqueous solution of glutaraldehyde (GA) specially purified for use as an electron microscopy fixative or other sophisticated use (all – from "Sigma–Aldrich Chemie", Germany), and glycerol of domestic production. Acetylcholine chloride (AChCl) (purity ≥ 99%) ("Sigma–Aldrich Chemie", Germany), was used as a substrate. Solutions of trichlorfon (analytical standard) ("Riedel-de Haën", Germany) and aflatoxin B1 (purity ≥ 98%) ("Sigma–Aldrich Chemie", Germany), cationic surfactant benzalkonium chloride (purity ≥ 95.0%) ("Fluka", Sweden), solutions of heavy metal nitrates (domestic production), solutions of crystalline α-solanine and α-chakonin from sprouts of *Solanum tuberosum* ("Sigma–Aldrich Chemie GmbH", Germany) were used as inhibitors.

Solutions of pyridine-2-aldoxime methyl iodide (PAM-2) (purity ≥ 98%) and ethylene diamine tetra acetate (EDTA) (analytical standard) ("Sigma–Aldrich Chemie", Germany) served as reactivators.

Compounds for preparation of buffers and other inorganic substances used in the work were of domestic production and had purity higher than 98%.

2.2. Conductometric transducers

The conductometric transducers (Fig. 1), used in this work, were produced according to the authors' recommendations at the Lashkarev Institute of Semiconductor Physics (Kyiv, Ukraine). They were 5 mm × 30 mm in size and composed of two identical pairs of gold interdigitated electrodes deposited on a ceramic base. The sensitive surface of each electrode pair was approximately 1.0 mm × 1.5 mm. The width of the transducer fingers and the distance between them were 20 mm. The conductometric transducers were connected to the measuring setup, which is described in detail in previous works [12,13].

2.3. Preparation of bioselective elements

A biomembrane was formed by the method of enzyme immobilization developed in our laboratory [14]. For preparing working bioselective membranes, a solution consisting of 1% acetylcholinesterase, 4% BSA and 10% glycerol in 20 mM phosphate buffer, pH 6.5, was used. The mixture for the reference membrane consisted of 5% BSA and 10% glycerol in the same buffer. After deposition of the prepared solutions on working surfaces of conductometric transducers, the latter were placed in saturated glutaraldehyde vapor for 20 min, and then kept for 5 min in air at room temperature. Before using in a biosensor, the membranes were washed with the buffer solution from excess of unbound components.

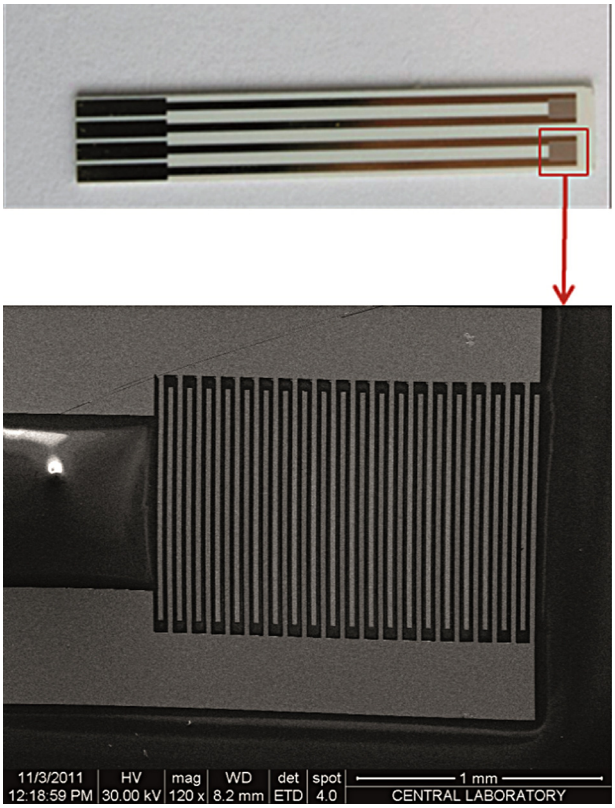

Fig. 1. General view of conductometric transducer and microimages of the gold interdigitated electrodes obtained with a scanning electron microscope.

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2.4. Measuring procedure

Measurements were carried out in 5 mM phosphate buffer, pH 6.5, at room temperature in an open cell under constant stirring. The concentrations of substrates in the working cell were obtained by an addition of aliquots of their stock solutions. To perform the inhibitory analysis, the responses to the substrate concentration were measured before (A_0) and after (A_i) inhibition. The method of inhibition of the bioselective element was varied depending on the type of inhibition (reversible or irreversible). In case of reversible inhibition (the scheme is shown in Fig. 2a), the inhibitor is added directly to the working cell; for the repeated procedure only washing with the working buffer is needed. At irreversible inhibition (Fig. 2b), after obtaining the response to the substrate (A_0), the biosensor is incubated in the inhibitor solution for some time, then washed from the inhibitor excess and the response to the substrate (A_i) is measured. For the repeated procedure, the biosensor is incubated in the solution of reactivators. The research on the reactivation is described in detail previously [12,15]. The residual enzyme activity R was evaluated by the formula: $R\% = (A_i/A_0) \times 100$, where A_0 – activity without inhibition, A_i – activity in the presence of inhibitor or after exposure to inhibitor. The residual activity, in turn, is proportional to the concentration of toxic substances in the sample.

All experiments were carried in two or three series. Non-specific changes in the output signal related to fluctuations of temperature, pH of environment, etc., were suppressed and avoided due to the differential mode measurements.

3. Results and discussion

The functioning of AChE biosensor is based on the following enzymatic reaction:

AChE

$$CH_3)_3N^+CH_2{-}CH_2{-}O{-}C(O){-}CH_3 + H_2O \rightarrow (CH_3)_3N^+CH_2{-}CH_2{-}OH + CH_3{-}COO^- + H^+ \quad (1)$$

During the enzymatic Reaction (1), the catalytic action of acetylcholinesterase splits acetylcholine into choline and acetic acid. The latter dissociates into an acid residue and a proton, the local concentration of ions in the working membrane increases, thus, the conductivity of the solution in the near-electrode region changes, which is registered by a conductometric transducer [16].

The concentration of toxic substances is determined by inhibitory analysis, which is based on the measurement of sensor responses to the substrate before and after inhibition of sensor by the toxicants [17] and calculation of residual activity of the enzymatic membrane according to the following equation:

$$R\% = \frac{A_0}{A_i} \times 100,$$

where A_0 is an activity without inhibition, A_i is an activity in the presence of inhibitor.

The first stage of this work was to determine optimal conditions of AChE immobilization onto the transducer surface, and optimization of main analytical characteristics of the biosensor. First, an effect of immobilization time on the functioning of AChE sensors was investigated. The values of responses of biosensors with membranes, immobilized in saturated GA vapor for different time, were measured. It was found that the optimal time of immobilization of AChE-based bioselective element was 20 min.

The next subject was an influence of the membrane activity on the biosensor performance. The changes of this parameter were modeled varying AChE concentration in the bioselective membrane. A number of biosensors with membranes based on AChE of

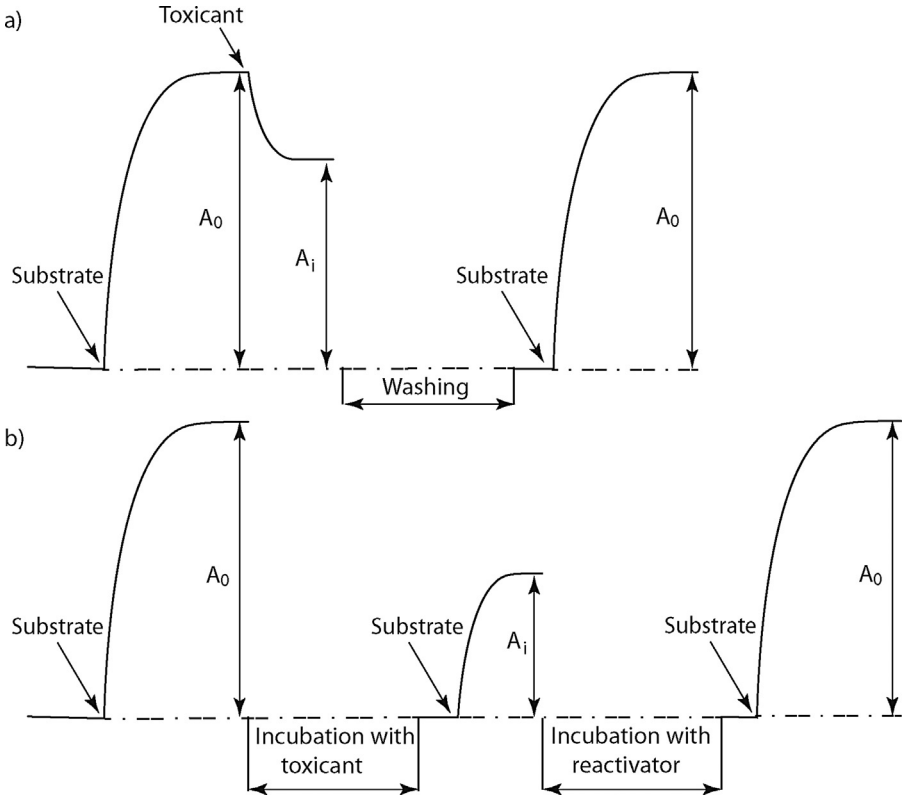

Fig. 2. Scheme of inhibitory analysis at reversible inhibition (a) and irreversible inhibition (b).

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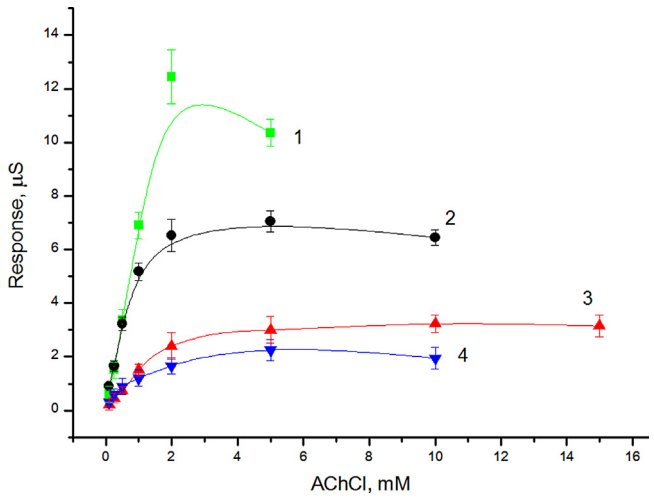

Fig. 3. Calibration curves of biosensors with different concentration of AChE in bioselective membrane (1–5%, 2–1%, 3–0.5%, 4–0.05%). Measurements were performed in 5 mM phosphate buffer, pH 6.5.

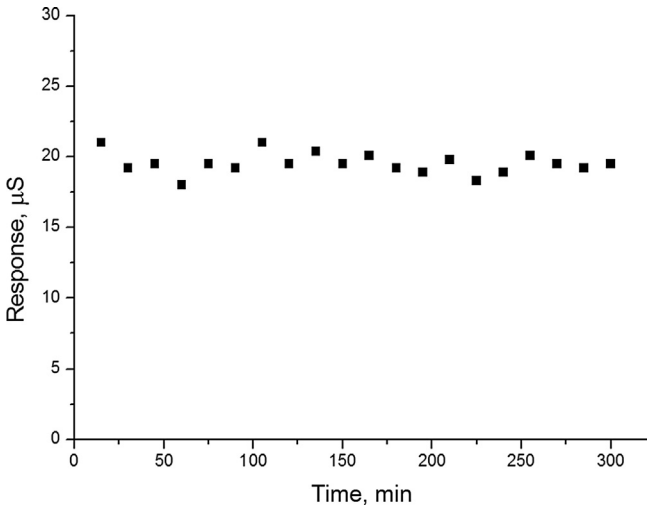

Fig. 4. Reproducibility of responses of conductometric biosensor based on immobilized acetylcholinesterase. Measurements were carried out in 5 mM phosphate buffer, pH 6.5, at room temperature, substrate concentration – 1 mM.

different concentrations (5%, 1%, 0.5%, 0.05%) were prepared and calibration curves, i.e., dependence of responses value on AChCl concentration, were plotted for all the biosensors obtained (Fig. 3).

As seen, the most sensitive was the biosensor based on 5% AChE (curve 1), that with 1% AChE demonstrated 1.5–2 times less responses. However, just the membrane with 1% AChE was selected for further work since its usage in biosensors, on one hand, ensures sufficient sensitivity, and on the other, promises in future to reduce the analysis cost. Use of lower enzyme concentrations (0.5% and 0.05%) results in a significant decrease in the biosensor sensitivity (curves 3 and 4). In the optimal case (1% AChE), the saturation of the biosensor was observed at AChCl concentration higher than 3 mM.

The next phase was optimization of the concentration of acetylcholine chloride (AChCl) as a substrate for inhibitory analysis.

Among the toxic substances inhibiting AChE, there are both irreversible (organophosphorus pesticides, heavy metals, etc.) and reversible (mycotoxins, glycoalkaloids, surfactants, etc.) inhibitors. The mechanisms of action of various inhibitors differ; correspondingly, optimal working concentrations of the substrate are diverse.

If inhibition is irreversible, it is necessary to choose the AChE concentration so that the biosensor sensitivity to the toxicant will be maximum. It means that each enzyme molecule should be mostly involved in the process of substrate conversion into the final product, which would result in changes in conductivity and generation of maximum response. It is possible at the enzyme saturation with substrate [15]. In our case, saturation was observed at 3 mM, thus in the further experiments on irreversible inhibition, 3 mM of AChCl was used.

If the inhibition process is reversible, it is necessary to choose the substrate concentration within the linear range of the calibration curve of the used biosensor so that the level of enzyme inhibition by the toxicant was high enough [18]. The optimum substrate concentration was chosen separately for each toxic substances. For this, the inhibition of substrate of different concentrations was performed using the toxicant of the same concentration. In the case of aflatoxin B1 determination, the high sensitivity to aflatoxin B1 was founded for AChCl concentration from 0.25 to 1 mM [19]. 1 mM AChCl was chosen as a working substrate concentration for the aflatoxin B1 determination, since large enough values of both response and inhibition level were observed at this concentration.

Working with irreversible inhibitors when the procedure consists of obtaining the biosensor response to the substrate, the biosensor incubation in the toxicant solution and subsequent receiving the response for the second time, it is necessary to eliminate an influence of the error in signal reproduction. For this purpose, one of the most important biosensor characteristics, the operational stability, was tested. Throughout one working day, the biosensor responses to the same substrate concentration (1 mM AChCl) were measured with 15-min intervals, during which the biosensor was kept in the working buffer with constant stirring. The results (Fig. 4) evidently show that the biosensor is characterized by quite high signal reproducibility with a relative standard deviation of less than 2.5%. Thus, it seems reasonable to assert that a remarkable change in the response can depend on biochemical reactions and not on a lack of biosensor stability. Furthermore, we have studied the biosensor stability during one month. In case of storage in working buffer in the fridge, the responses decreased gradually and were at least 90% at the end of the month. Thus, the biosensor should be calibrated after storage in order to obtain the most accurate results.

In the case of irreversible inhibition, the time of biosensor incubation in the solutions with toxic substances is an important parameter. Therefore, a series of experiments were conducted to determine the optimum time of the enzyme contact with toxic analytes. The time was determined to be 20 min, since during this period the biosensor lost 20–80% of its initial activity, which is an informative indicator. The level of reversible inhibition depends neither on the incubation time nor on the sequence of injection of substrate and toxicant into the working cell [18].

The next step was to study an effect of different classes of toxic substances, organophosphorus pesticides, heavy metal ions, mycotoxins, surfactants and glycoalkaloids, on the AChE residual activity in the biosensor. The calibration curves of dependence of the biosensor residual activity on the concentration of different toxicants were obtained (Fig. 5).

In Fig. 5, curve (1) is a calibration curve for determination of trichlorfon, as a pesticide example. The linear range of determination is seen to be 25–300 μM. The linear determination range for Cu^{2+} ions (calibration curve 2) is 5–1200 μM. They are quite acceptable values, however, other enzyme systems, such as urease-based [20] or three-enzyme (glucose oxidase/mutarotase/invertase) based systems [12], are known to be more sensitive to heavy metal ions. Curve (3) shows relatively high biosensor sensitivity to

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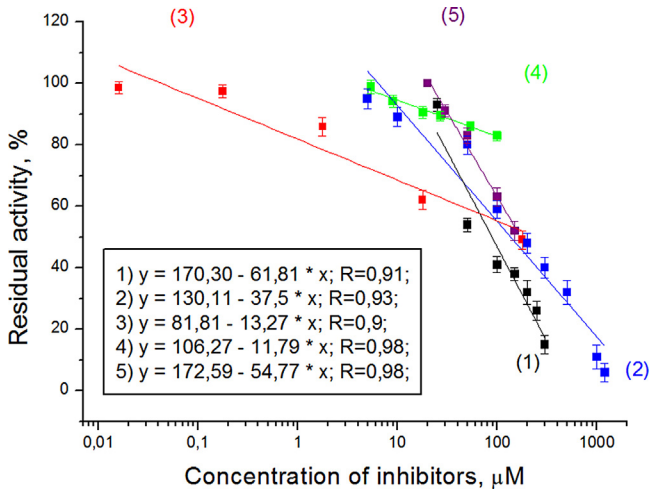

Fig. 5. Dependence of residual activity of bioselective element of AChE-based biosensor on concentration of trichlorfon (1), Cu^{2+} ions (2), aflatoxin B1 (3), chloride benzalkonium (4), and α-solanine (5). Measurements were performed in 5 mM phosphate buffer, pH 6.5, at room temperature.

aflatoxin B1 in the linear range 0.02–170 μM. When analyzing the linear range of determination of benzalkonium (curve 4), it can be put into question the feasibility of using AChE as a bioselective element for the surfactants measurement. Saturation by the inhibitor was observed at the 75–80% level of residual activity, and it is far from enough to provide sufficient sensor sensitivity to this class of substances in the required concentration range. The glycoalkaloid solanine can be determined by AChE-based biosensor beginning with the concentration of 20 μM (curve 5), whereas the concentration of 1–2 μM should be measured. At the same time, the biosensor based on butyrylcholinesterase (BuChE) has a higher sensitivity, which is sufficient to work with GA [21].

Thus, analyzing the findings, a conclusion can be made that AChE biosensor is an efficient tool for the quantitative analysis of aflatoxin B1 or for the determination of total toxicity of the sample. In principle, it is also possible to use this biosensor for identification of heavy metals, steroid glycoalkaloids, organophosphorus pesticides and surfactants, however, it demonstrates a lack of sensitivity, thus, its use for quantitative analysis is less rational.

Aflatoxin B1 is a very strong neurotoxin with high affinity to AChE. According to the recent research, aflatoxin B1 inhibits the AChE molecule by binding at the peripheral site, located at the active site entrance (at the tryptophan residue) [22]. From the physiological point of view, reversible interaction of the toxins with AChE is useful: one molecule of aflatoxin can inhibit several AChE molecules at different times and cause disorders in several parts of the nervous system. According to the literature data, the AChE-based biosensors are more sensitive to aflatoxin than to other inhibitors [4]. However, there are still many information gaps about the mechanism of aflatoxin action.

During the work, the membrane working activity (the response to substrate) was revealed to recover after inhibitory analysis in a diverse way depending on the toxic substances, which are present in the sample tested. For example, even for washing the biosensor from reverse inhibitors, which commonly are removed from the membrane by routine washing in the working buffer, the washing procedures differ. An entire washing of the membrane exposed to alkaloids and aflatoxins lasted about the same time as the response, i.e., 3–5 min, whereas the washing of the membrane exposed to surfactants lasted about 30–40 min.

The sensor response after exposure to irreversible inhibitors can be restored to the initial values only by using special materials, the reactivators of enzymes. To reactivate cholinesterases, the substances pyridine-2-aldoxime methyl iodide (PAM-2) and ethylene diamine tetra acetate (EDTA) are most commonly used. The study on reactivation demonstrated some specificity of reactivators, which was confirmed by a series of experiments, schematically shown in Fig. 6.

Fig. 6b shows the opposite picture – enzyme inhibition by Cu^{2+} ions leads to a decrease in activity to 68%. Reactivation in PAM-2 has no effect, but the use of EDTA results in the 100% recovery of the biosensor response to AChCl.

Thus, by using different schemes of reactivation of irreversible and reversible inhibitors, it is possible to differentiate the identification of different classes of toxicants.

As noted above, an important parameter of irreversible inhibition is the time of incubation. Equally important is the time of reactivation. Therefore, experimentally, it was determined that the optimum time of biosensor incubation in the solution of reactivator results in entire or maximal possible recovery of the membrane activity.

Table 1 summarizes the experimental results obtained in the investigation on sensitivity of AChE-biosensors to various toxic substances.

As seen from Table 1, the AChE sensor has the highest sensitivity to mycotoxins (16 nM), as well as sufficiently large linear range of determination. The reactivation occurs fast enough and is a result of ordinary washing of a bioselective element with a working buffer. The sensitivity to organophosphorus pesticides was

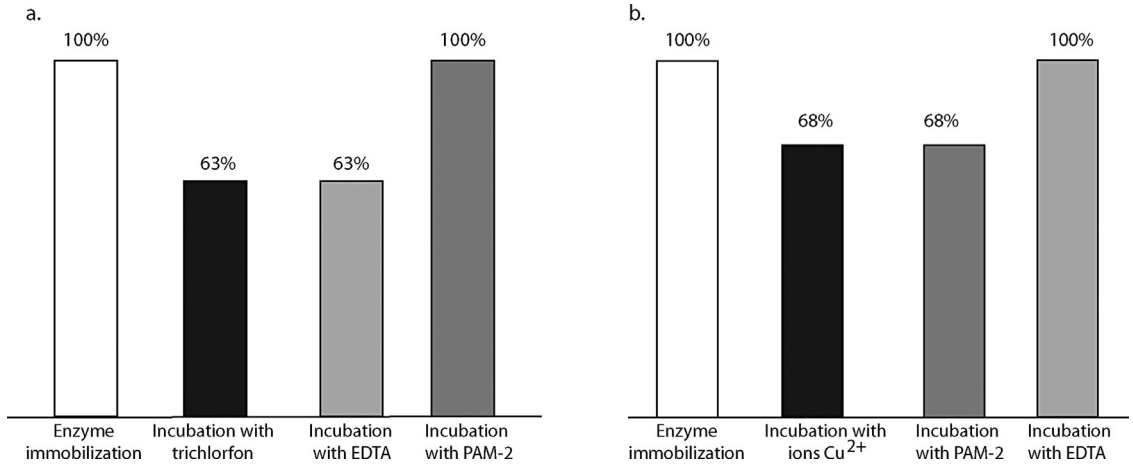

Fig. 6. Scheme of experiment, which demonstrated selectivity of reactivation process.

Table 1
The analytical characteristics of AChE-biosensors for the inhibitory analysis of toxic compounds of different nature.

Toxicant	Type of inhibition	Limit of detection (μM)	Linear range (μM)	Reactivator	Time of reactivation (min)	Number of reactivation cycles
Mycotoxins	Reversible	0.016	0.02–170	Phosphate buffer	3–5	>50
Organophosphorus pesticides	Irreversible	25	25–300	PAM-2	20	5
Surfactants	Reversible	5.4	5–100	Phosphate buffer	30–40	>50
Heavy metal ions	Irreversible	5	5–1200	EDTA	30	4
Glycoalkaloids	Reversible	20	20–140	Phosphate buffer	3–5	>150

1000 times lower (25 μM); in this case, the reactivation lasts 30 min. PAM-2 as a reactivator was used at least 5 times, which is a good result for determination of an irreversible inhibitor. The linear range of determination of surfactants is rather narrow, but the number of reactivation cycles is sufficient. Reactivation of surfactants is slower than that of mycotoxins and glycoalkaloids, which are also reactivated using the working buffer. The linear range of determination of heavy metal ions is quite wide. Reactivation with EDTA lasts 30 min. The largest number of reactivation cycles can be carried out for glycoalkaloids. Reactivation occurs fast, washing with the working buffer is used.

An analysis of these data permits us to offer a method of selective determination of toxic substances in aqueous samples using the AChE based biosensor. The essence of this method can be represented as a block diagram (Fig. 7) demonstrating the analysis algorithm.

After the sampling, the aqueous probe is filtered to remove large particles which can damage the bioselective membrane

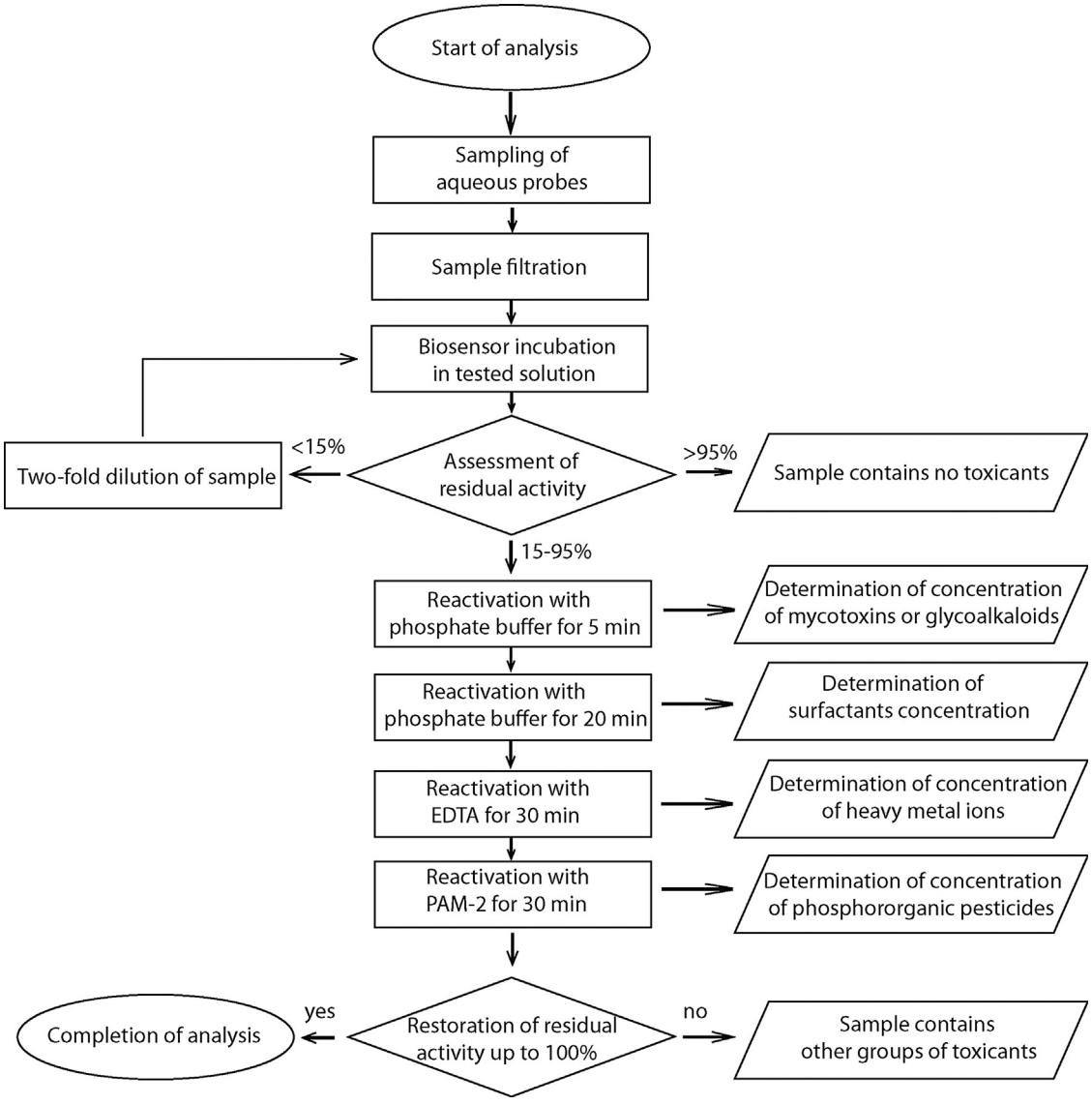

Fig. 7. Block diagram of inhibitory analysis of toxic substances by AChE-based biosensor using selective reactivation of bioselective element.

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mechanically. The membrane working activity is determined and taken as 100%. Next, the biosensor is incubated in the toxicant solution, and the residual activity of the enzyme membrane is analyzed. If it is higher than 95%, a conclusion can be made that the concentration of toxic substances in the sample is too small to be registered by biosensors. If the residual activity is lower than 15%, the sample is diluted until the residual activity is in the range of 15–95%. To determine the toxic composition, the biosensor is first washed with the working buffer. If a complete recovery of the membrane working activity is observed during 5–10 min, it can be an evidence of the presence of reversible inhibitors in the sample, either glycoalkaloids or mycotoxins, depending on the sample origin. If recovery requires more prolonged washing in the working buffer (20–30 min), it indicates the presence of surfactants in the sample. If after 30–40-min washing the biosensor response is not restored to 95%, then the presence of irreversible inhibitors in the tested solution can be stated.

To clarify the class of irreversible inhibitors, the selective reactivation is carried out. First, the biosensor is reactivated using EDTA to avoid the effect of heavy metal ions on the enzyme membrane. If this stage results in recovery of the biosensor activity, it is possible to assume that heavy metal ions are present in the sample. If the activity is not yet restored to 100%, the biosensor is reactivated with PAM-2 to detect organophosphorus pesticides.

Thus, if after the assay is completed and all phases of reactivation are performed, the activity of bioselective element is entirely recovered, it is possible to determine the toxic composition of the sample analyzing the membrane activity at each stage of reactivation. If the activity is recovered partly, the sample likely contains toxic substances of some classes other than mentioned above. However, the total toxicity of the sample can be determined, and if necessary, the additional analysis may be used for further study.

Several disadvantages of the proposed biosensor should be indicated. If an analyzed sample is a mixture of inhibitors of the same class (i.e., several heavy metal ions or several surfactants), the proposed biosensor cannot specify the inhibitors within the certain class and will evaluate the total concentration of inhibitors of each class. Furthermore, it is difficult to estimate the time of one analysis. In some cases (reversible inhibition by aflatoxin), the analysis lasts only 10 min, whereas in case of organophosphate pesticides, the reactivation consists of several steps and total time of analysis increases up to 2 h. Finally, sensitivity of the proposed biosensor to certain toxic compounds is worse in comparison with the previously developed biosensor, but this shortage is compensated by the versatility of the proposed biosensor and the possibility of distinguishing among different inhibitors.

5. Conclusions

The conductometric biosensor based on immobilized acetylcholinesterase is developed for the inhibitory determination of toxic substances of different nature. The optimal time of enzyme immobilization on the surface of conductometric transducer in saturated GA vapor was 20 min. The optimal concentrations of substrates for inhibitory analysis are selected; depending on the type of inhibition and the class of toxins, they were 1 mM and 3 mM AChCl for reversible and irreversible inhibition, respectively. The sensitivity of the developed biosensor to the toxicants of different classes was tested. The linear range of determination of trichlorfon was 25–300 μM, for Cu^{2+} ions – 5–1200 μM, for aflatoxin B1 – 0.02–170 μM, for chloride benzalkonium – 5–100 μM, for solanine – 20–150 μM. The feasibility of application of this biosensor for selective detection of different toxic agents was analyzed. It is found that AChE-based biosensors can be used to identify different groups of toxins, but preferably to determine aflatoxin or to detect total toxicity of the sample. A new approach is proposed for selective analysis of different toxic compounds in the sample by a single AChE-based biosensor applying additional stages of enzyme reactivation of bioselective membrane after every measurement.

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