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Enzymatic biosensors for determination of pesticides

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The most recent results of research and development in the field of enzyme-based biosensors for determination of pesticides are presented. The possibilities of pesticide quantification *via* enzyme inhibition by pesticides, hydrolysis of pesticides by various enzymes or redox reactions involving enzymes are discussed. Particular attention is paid to the use of single enzymes both in the soluble and immobilized forms and to application of combinations of several enzymes catalyzing successive reactions to improve the determination. Among enzyme-based biosensors for pesticide determination, analytical systems successfully utilizing enzymes within microbial cells are surveyed. The recently achieved characteristics of enzyme-based biosensors are presented and further prospects for their development are discussed. The bibliography includes 100 references.

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I. Introduction

The production volumes demanded from modern agriculture could not be attained without using chemical protection agents — pesticides. An estimate of average annual consumption of pesticides in 93 countries considering data of the Statistics Division of the Food and Agriculture Organization of the United Nations (FAOSTAT, http://faostat3.fao.org/), the United States Department of Agriculture [1] and the Ministry of Agriculture and Farmers Welfare of India (http://www.cibrc.nic.in/mup.htm) provides the conclusion that > 4.3 million tonnes of pesticides are used every year all over the world. The level of pesticide consumption is quite stable for a long period of time, despite any financial and climatic ups and downs. The highest percentage is accounted for by China (> 40%), the next largest consumers are European Union (12% – 14%), Brazil (6% – 7%) and the USA (5% – 6%). The range of pesticides used is highly variable among the countries, even for the countries located in the same region or in the same climatic area. Nevertheless, in general, the most consumed types of agents are fungicides and herbicides, which can reach in total 60% – 80% of all used pesticides.

The growth of Earth's population, together with gradual propagation of pest resistance to long-used plant protection agents stimulate the development and commercialization of new pesticides. The formerly used pesticides, which are often more toxic for humans than the newly developed ones, are gradually withdrawn from the market and/or become subject to more and more stringent restrictions on their levels in products and in the environment. This, in turn, brings about the need for thorough monitoring of these compounds in diverse specimens by means of highly efficient determination techniques.

The commonly accepted chromatographic, spectrometric and electrochemical methods of analysis, including advanced versions, possess obvious benefits for laboratory detection of pesticides.[2] Biosensors, which are considered in this review, advantageously supplement and extend the scope of such analysis. Biosensors for pesticides, in particular, enzyme-based biosensors are the subjects of numerous papers. The key principles that underlie the design of these biosensors are exhaustively covered in several recent reviews.[3 – 7] However,

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these publications often lack information about the currently existing biosensors available on the mass market. This considerably decreases the practical value of these reviews and does not allow one to evaluate in full measure the progress of science and technology in this field. Furthermore, the method for the formation of biosensors, which is a rather important issue, does not receive adequate attention. Meanwhile, for example, the use of a particular method for enzyme immobilization may predetermine its biochemical properties, thus affecting the analytical characteristics of the final biosensor.

The purpose of the present review is to analyze the key results of research published in 2010–2015 (and some papers of 2016) concerning the development of biosensor systems that contain enzymes as recognition elements and are applicable for determination of widely used pesticides. The attention is focused on the practical aspects of commercialization of these devices. Promising trends of further studies dealing with new enzymatic biosensors for the detection of pesticides are formulated. In this analysis, it was of particular interest to consider new ideas coming from successful works in other branches of science and technology (in particular, modern approaches to enzyme immobilization) and ways to implement them in the field of our interest.

Note that in this review, we did not aim to discuss the enzyme immunoassay methods for analysis of pesticides, in particular, the works dealing with molecular design of antibodies.[8] The reason is that in these methods, the function of the enzyme label is to enhance the analytical signal rather than to generate the signal upon interaction of an enzyme with an analyte, which implies a significant difference between the operation principles of these biosensors and enzymatic biosensors. The state of the art of research in enzyme immunoassay methods as applied to pesticides is covered in a number of papers.[9–13] In addition, the considerable progress in the development of analytical systems based on molecularly imprinted polymers,[14] which are beyond our scope either, should be mentioned.

Most enzymatic biosensors are now meant for detection of pesticides in various biological specimens (agricultural products, biological fluids, for example, blood, *etc.*) and environmental objects (natural water sources, soil extracts and so on), which contain a variety of other chemicals together with the analyte.[15] The problem is the rapid, selective and efficient quantification of a pesticide. Particularly enzymatic biosensors are, in principle, applicable to solving this problem, because enzymes are high-performance protein catalysts possessing unique properties: high sensitivity and selectivity of catalytic action towards specific substrates, the ability to rapidly respond to the adverse action of some inhibitors under conditions (temperature, pH, chemical composition) inherent in natural objects and processes taking place *in vivo*.

Various pesticides are either actively used in practice (in agriculture, in textile and wood industry, in household and in other areas of human activity) or have already been banned, but it is still necessary to detect them in different specimens. They can be determined by means of enzymatic biosensors operating on different principles: some are based on the inhibition of enzyme action by the pesticide, while the other operate *via* enzymatic catalysis of chemical reactions (oxidation or hydrolysis) involving pesticides.[16] This process results in the generation of an analytical signal, which can be reliably detected after amplification and/or transduction.

The established maximum permissible levels of pesticides (Table 1) often impose high requirements on the sensitivity of analysis;[17] therefore, the goal of most modern studies is to develop an analytical system with reasonably high characteristics. This can be done by varying both the recognition element (enzyme) and the

Table 1. Examples of pesticides and their permissible levels in food products.

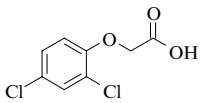
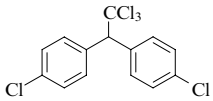
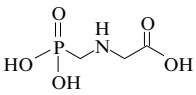
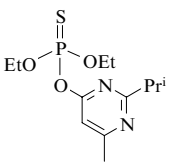
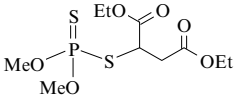
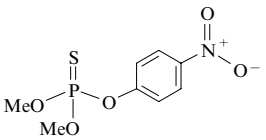
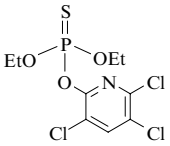
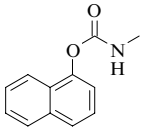
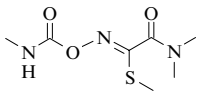
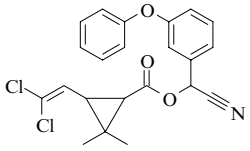
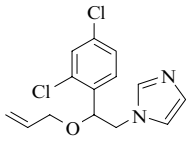
Pesticide	Structural formula	Maximum residue level[a] /mg kg^{-1}	Tolerable daily intake /μg day^{-1} kg^{-1}
2,4-D (2,4-dichlorophenoxyacetic acid)		0.01–5[b]	10
DDT [1,1,1-trichloro-2,2-bis(4-chlorophenyl)-ethane]		0.02–5	10
Glyphosate [*N*-(phosphonomethyl)glycine]		0.05–15	0–1000
Diazinon [*O*,*O*-diethyl *O*-(2-isopropyl-6-methylpyrimidin-4-yl) phosphorothioate]		0.02–2	0–5

Table 1 (continued).

Pesticide	Structural formula	Maximum residue level [a] /mg kg^{-1}	Tolerable daily intake /μg day^{-1} kg^{-1}
Malathion [*O*,*O*-dimethyl *S*-{1,2-di(ethoxycarbonyl)ethyl} phosphorodithioate]		0.01 – 10	0 – 300
Methyl parathion [*O*,*O*-dimethyl *O*-(4-nitrophenyl) phosphorothioate, MPT)		0.05 – 5	3
Chlorpyrifos [*O*,*O*-diethyl *O*-(3,5,6-trichloropyridin-2-yl) phosphorothioate]		0.01 – 20	0 – 10
Carbaryl [1-naphthyl-*N*-methylcarbamate]		0.02 – 25	0 – 8
Oxamyl [*N*,*N*-dimethyl-2-methylcarbamoyloximino-2-(methylthio)acetamide]		0.02 – 5	9
Cypermethrin [α-cyano-3-phenoxybenzyl 3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropanecarboxylate]		0.01 – 10	0 – 20
Imazalil [(*RS*)-1-[2-allyloxy-2-(2,4-dichlorophenyl)ethyl]-1*H*-imidazole]		0.01 – 5	30

[a] The ranges of maximum residue levels and tolerable daily intakes for different food products are given according to the Codex Alimentarius FAO/WHO (http://www.fao.org/fao-who-codexalimentarius/). [b] The sum of 2,4-dichlorophenoxyacetic acid and its salts and esters expressed in equivalents of the major active component.

method used to amplify and detect the signal. A lucky combination of these constituents (recognition, amplification and detection) of biosensors provides very high sensitivity of analysis (down to ng litre^{-1}). Relying on accumulated empirical data and using modern physicochemical methods, it has now become possible to develop enzymatic biosensors for particular analytical applications; this formed the basis for the design of commercially available types of these biosensors and gave rise to increasing interest of potential consumers in these devices.

II. Operation principle of enzymes for determination of pesticides

Enzymatic biosensors used to determine pesticides can be based on various enzymes; as a rule, the catalytic action of these enzymes is realized *via* hydrolysis or oxidation of pesticides, which function as enzyme substrates, or *via* enzyme inhibition upon the interaction with pesticides. Below we consider examples of analytical determination of pesticides by means of enzymatic biosensors with various operation principles and the enzymes used most widely in the recent research along this line.

II.1. Determination of pesticide concentrations with enzyme inhibition

The practical use of many pesticides is aimed at inactivation of enzymes of different biological origin. Detailed analysis of the mechanisms of enzyme inhibition by pesticides has been reported in a number of papers (see, for example, Refs 18 and 19). The knowledge of these mechanisms allows the use of appropriate enzymes for the detection of pesticides, the degree of inhibition being, as a rule, proportional to the concen-

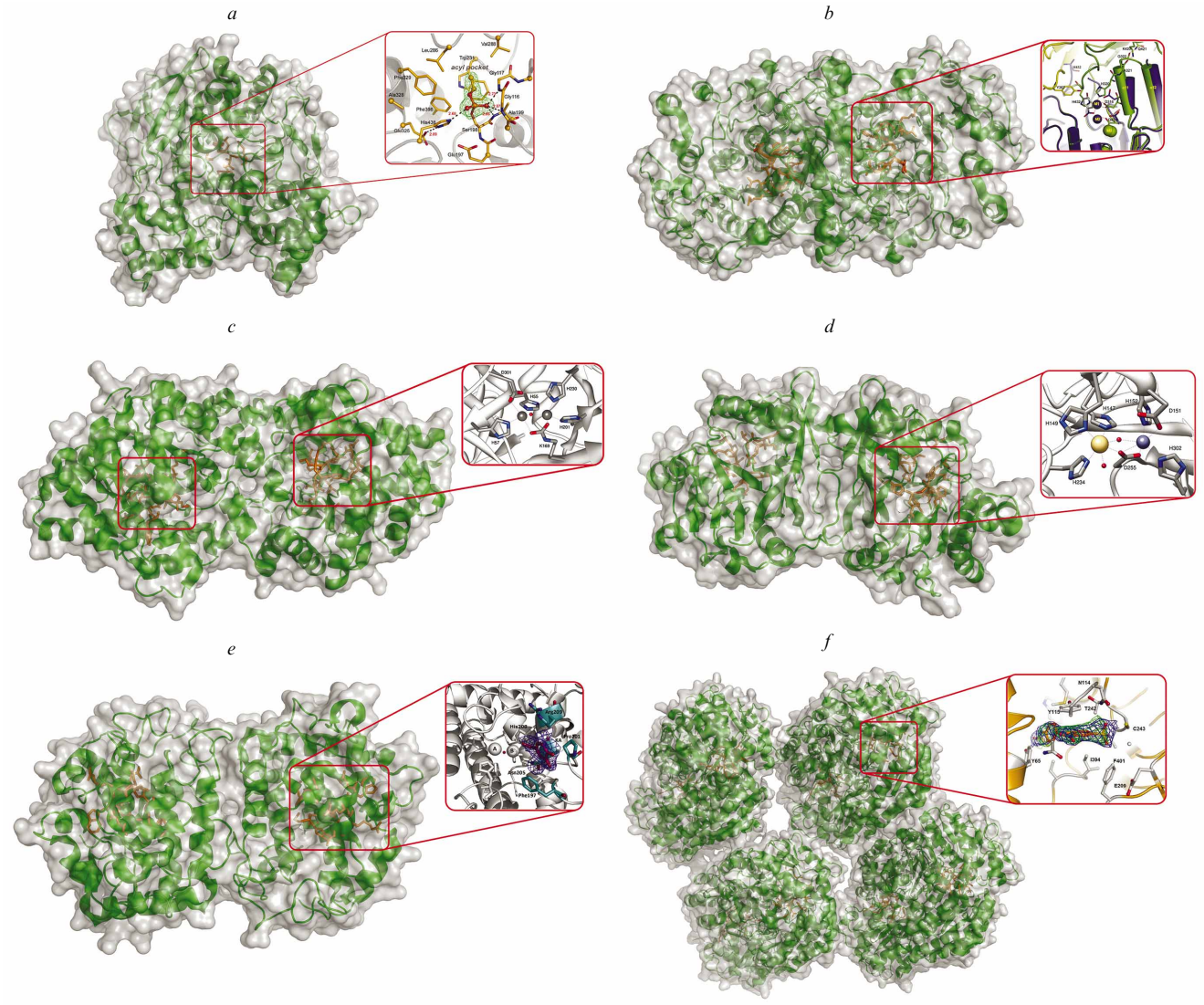


Figure 1. Structure of acetylcholinesterase (RCSB PDB: 2WG2) (*a*),[20] dimer of alkaline phosphatase (RCSB PDB: 4KJG) (*b*),[21] dimer of organophosphorus hydrolase (RCSB PDB: 1QW7) (*c*),[22] dimer of methyl parathion hydrolase (RCSB PDB: 1P9E) (*d*),[22] dimer of tyrosinase (RCSB PDB: 3NM8) (*e*),[23] octamer of aldehyde dehydrogenase (RCSB PDB: 4H80) (*f*).[24]
The active site configurations were taken from appropriate sources with permission of Elsevier and Wiley-VCH. The molecular surface was calculated by authors of the present review using the PyMOL program, version1.7.6.0, and known amino acid sequences and open access crystallographic data.

tration of a particular pesticide. The structure of the enzymes receives much less attention (Fig. 1). Meanwhile, considering the enzyme structure could enable a rational approach to the design of enzymatic biosensors for particular tasks including, for example, point chemical modifications of protein molecules, various types of their additional stabilization and so on.

The greatest number of pesticides inhibit, to one or another extent, acetyl- and butyrylcholinesterases, the former of which is responsible for neurotransmission in living organisms (see Fig. 1 *a*).[20] The active site of cholinesterases contains a serine residue, which is necessary for these enzymes to exhibit catalytic activity towards acetyl- and butyrylchlolines. Phosphorylation of this serine residue with, for example, organophosphorus compounds results in almost irreversible inhibition of the enzyme. Therefore, the decrease in the enzyme activity upon the contact with a pesticide can be used to detect various inhibitors with a high sensitivity, because in this case, one and the same enzyme acts as both a sensing element and signal amplifier. The modern micro- and nanomechanical devices even do not require the signal amplification due to direct recording of the interaction between the enzyme and the inhibitor (see below, Section VI.2). High binding affinity between cholinesterases and some pesticides in combination with highly sensitive procedures for determination of enzymatic activity make it possible to detect exceptionally low concentrations of inhibitors; hence, these enzymes are excellent objects for investigation and for development of numerous biosensors.

Another type of enzymes that are inhibited by pesticides are acid and alkaline phosphatases (see Fig. 1 *b*).[21] Phosphate monoesters are natural substrates for these enzymes; therefore, phosphate di- and triesters (organophosphorus pesticides mentioned above) are often competitive phosphatase inhibitors, as their active site is poorly adapted for catalyzing the hydrolytic reaction of these substrates.

Like cholinesterases, phosphatases act simultaneously as recognition elements and signal amplifiers. However, since in this case, the enzyme inhibition is reversible, the sensitivity of determination of inhibitors using phosphatases as recognition elements is always substantially lower than with cholinesterases.

II.2. Enzyme-catalyzed hydrolytic reactions

One more large group of enzymes used in the development of biosensors are hydrolases (see Fig. 1 *c*,*d*)[22] that are able to hydrolyze some pesticides; the decomposition products thus formed are then detected *via* generation of a signal of a particular intensity. For example, the products of hydrolysis of organophosphorus pesticides such as methyl parathion and chlorpyrifos can be determined by spectrophotometry, because the resulting compounds have a characteristic absorption spectrum with a maximum in the blue-violet visible range and in the near-UV range. However, there are only few compounds with such type of absorption among the hydrolysis products of pesticides or these compounds are difficult to distinguish in the analyzed media, which contain other compounds interfering with the specific determination. Therefore, other methods for detecting the analytical signal are often employed.

The enzymatic hydrolysis of pesticides is usually accompanied by the formation of charged molecules and by a change in the pH of the reaction medium; this can be detected directly. The utilization of electrochemical measurements for determination of products formed in enzymatic reactions may increase the sensitivity of analysis. However, it should be noted that such hydrolytic enzymes usually react with a small range of compounds of a particular class, which precludes their use for the simultaneous determination of different classes of pesticides (*e.g.*, organophosphorus and organochlorine compounds and carbamates). Nevertheless, such hydrolases can be applied in combination with other enzymes, for instance, with cholinesterases, in order to enhance the analytical characteristics of the system as a whole; this issue is discussed below.

II.3. Enzyme-catalyzed redox reactions

Apart from hydrolases, oxidoreductases are also applicable in enzymatic biosensors for determination of pesticides (see Fig. 1 *e*,*f*);[23,24] however, only few oxidoreductases are able to catalyze reactions directly involving pesticides. The strategy where pesticides inhibit enzymes of this type is used much more widely. In this case, the operation principle of oxidoreductase-based biosensors is similar to that of analogous biosensors based on cholinesterases.

An undoubted advantage of using oxidoreductases as recognition elements is that their activity can be determined directly by electrochemical methods without participation of any mediators (intermediate electron donors or acceptors) owing to direct electron transfer between the enzyme and the electrode. Therefore, from the standpoint of potential practical application, redox enzymes may be much more promising than cholinesterases, despite the lower sensitivity to the given analytes.

III. Types of enzymatic biosensors for determination of pesticides in terms of the method of their preparation

The method for preparation of enzymatic biosensors is usually dictated by the subsequent model of their application. The simplest way is to use the proper enzymes in the soluble form; however, a more adaptable solution suitable for commercialization is to use biosensors based on immobilized enzymes. Furthermore, the use of enzymes in the immobilized form in biosensors has enabled the design of complex multicomponent (multi-enzyme) bioanalytical systems with specified characteristics.

III.1. Soluble biocatalytic systems

Initially, enzymes for determination of pesticides have been proposed exactly in the soluble form and, despite the considerable progress in the development of immobilized enzymatic biosensors, soluble systems are still quite popular (some examples are presented in Table 2). This is caused by a number of factors, among which the simplicity of preparation and application should be mentioned; therefore, if it is necessary to perform large-scale analyses of pesticides in various environmental specimens, the development and application of such enzyme products, which would provide primary information about the presence of a contaminant, are fully justified. For example, analytical systems of this type may be demanded for analysis of samples of coastal water and bottom sediments from the Baikal lake (within the framework of the Federal Target Programme 'Protection of the Baikal Lake and Socio-Economic Development of the Baikal Natural Area for 2012 – 2020') and samples of sea water from different depths and bottom sediments from the Caspian sea, which receives the inflow of pesticide-bearing water from agricultural lands of several countries [in terms of the 'Framework Convention for the Protection of the Marine Environment of the Caspian Sea (Tehran Convention)' signed in 2003].

Without dwelling on particular analytical characteristics of soluble enzyme forms used for determination of pesticides, in this Section, we would like to note that diverse enzymes and their derivatives[28 – 31,39] obtained by genetic modification are used for this purpose. Specifically, recombinant enzymes have aroused particular interest of researchers in recent years, because protein design resorting to genetic engineering may produce enzymes with desired characteristics. In the long view, this approach could markedly extend the analytical potential of biocatalytic systems; this trend would be even more pronounced in the case of biosensors based on immobilized enzymes.

Table 2. Examples of soluble enzyme forms applied for pesticide determination.

Enzyme	Determination method	Limit of detection [a]	Ref.
OPH (from *E. coli*)	Fluorescence of quantum dots	18 μg litre^{-1} MPT (in real specimens: down to 50 μg litre^{-1} or mg kg^{-1}, recovery: 96% – 117%)	25
	The same	15.8 μg litre^{-1} MPT (in real specimens: down to 100 μg litre^{-1} or μg kg^{-1}, recovery: 90% – 105%)	26
	Optical absorption of a fluoride-sensitive chromophore	18.4 μg litre^{-1} DFP	27
OPH-His_6 (from *E. coli*)	Fluorescence of the label (pyranine)	2 μg litre^{-1} PX, 10 μg litre^{-1} MPT	28
	The same	20 μg litre^{-1} PX, 50 μg litre^{-1} MPT, 50 μg litre^{-1} coumaphos (in real specimens: down to 50 μg litre^{-1}, recovery: 90% – 125%)	29
	Differential pulse voltammetry	0.15 mg litre^{-1} PT, 0.13 mg litre^{-1} MPT	30
	The same	0.14 mg litre^{-1} PX	31
Alkaline phosphatase (from bovine pancreas)	Fluorescence of β-naphthol	1.34 mg litre^{-1} fenitrothion (in real specimens: down to 10 mg litre^{-1}, recovery: 96% – 100%)	32
Trypsin (n.d.)	Fluorescence of quantum dots	40 ng litre^{-1} MPT (in real specimens: down to 0.2 μg litre^{-1} or μg kg^{-1}, recovery: 95% – 110%)	33
AChE (from eel)	Two-photon fluorescence of $NaYF_4$:Yb,Er nanoparticles	2 ng litre^{-1} MPT (in real specimens: down to 30 ng litre^{-1}, recovery: 96% – 110%)	34
	Fluorescence of DACM	1.5 ng litre^{-1} PX (in real specimens: down to 4 ng litre^{-1}, recovery: 94% – 110%)	35
AChE (n.d.)	Fluorescence and/or absorption of Rhodamine B	0.1 μg litre^{-1} carbaryl, 0.1 μg litre^{-1} diazinon, 0.3 μg litre^{-1} malathion, 1 μg litre^{-1} phorate (in real specimens: down to 0.1 μg litre^{-1}, recovery: 77% – 109%)	36
AChE (from eel), tyrosinase (from fungus)	Fluorescence of the label (FITC)	In real specimens: 0.6 μg litre^{-1} chlorpyrifos, 1.2 μg kg^{-1} pyrocatechol (recovery: n.d.)	37
AChE (from eel) in combination with choline oxidase (from *Alcaligenes* sp.)	Fluorescence of quantum dots	1 μg litre^{-1} dichlorvos (in real specimens: down to 170 μg litre^{-1}, recovery: 97% – 101%)	38
His_6-Z-S-OPH-Z (from *E. coli*) in combination with horseradish peroxidase	Chronoamperometry	7.56 mg litre^{-1} dichlofenthion	39

Note. n.d. means no data; OPH is organophosphorus hydrolase; OPH-His_6 is organophosphorus hydrolase with a hexahistidine tag at the C-terminus; AChE is acetylcholinesterase; DACM is *N*-(7-dimethylamino-4-methylcoumarin-3-ol)maleimide; FITC is fluorescein 5(6)-isothiocyanate; His_6-Z-S-OPH-Z is the mutant OPH containing a hexahistidine tag at the N-terminus, an α-helical leucine zipper domain (a similar domain is present at the C-terminus) and an unstructured glycine-rich domain for increasing the solubility. Test compounds: DFP is *O*,*O*-diisopropyl fluorophosphate, PX is paraoxon [*O*,*O*-diethyl *O*-(4-nitrophenyl) phosphate), PT is parathion [*O*,*O*-diethyl *O*-(4-nitrophenyl) phosphorothioate], coumaphos is *O*,*O*-diethyl *O*-3-chloro-(4-methyl-2-oxo-2*H*-chromen-7-yl) phosphorothioate, fenitrothion is *O*,*O*-dimethyl *O*-(3-methyl-4-nitrophenyl) phosphorothioate, phorate is *O*,*O*-diethyl *S*-[(ethylsulfanyl)methyl] phosphorodithioate, dichlorvos is *O*,*O*-dimethyl *O*-(2,2-dichlorovinyl) phosphate, dichlofenthion is *O*,*O*-diethyl *O*-(2,4-dichlorophenyl) phosphorothioate.

[a] In the case of testing of real specimens, the limit of detection or the minimum concentration of pesticides subjected to analysis and the recovery referred to that for the reference method (HPLC, GC/MS, *etc*.) are given.

Apart from the change in the physicochemical (optimal pH, substrate specificity, thermal stability), including catalytic (increase in the efficiency constant, *etc*.), characteristics of the target enzyme induced by point mutations of a recombinant protein, one more popular modification of enzymes is the introduction of an additional amino acid sequence (for example, hexahistidine one) at the N- or C-terminus of the protein molecule.[28 – 31, 39] This markedly facilitates the isolation and purification of such enzymes using preparative metal chelate chromatography, thus decreasing the labour intensity of the preparation of analytical systems of this type and, hence, reducing their cost.

III.2. Immobilized enzymes in the biosensing systems for determination of pesticides

Biosensors based on immobilized enzymes as recognition elements for the presence of pesticides in various media are now encountered most often. Along with the possibility to utilize various enzymes, it is possible to apply various immobilization techniques in the design of these biosensors. The choice of the technique is dictated by the model and conditions of analysis to be performed. There are three key techniques of enzyme immobilization that have already become classic — physical, chemical and affinity (mainly, metal chelate one).[40] Any of them can serve for the manufacture of efficient recognition elements of enzymatic biosensors for determination of pesticides (Table 3).

The physical immobilization of enzymes used as recognition elements implies the attachment of enzymes to some support *via* non-covalent interactions.[41 – 57] This can be done by adsorption on the support surface, absorption in the carrier pores, encapsulation and so on. This immobilization technique has some advantages, first of all, the ease of preparation and regeneration of stabilized enzyme. Among the drawbacks of enzymatic biosensors fabricated by this method, note low stability, which is due to the possibility of detachment of the recognition element from the matrix, for example, as a result of desorption. This induces a change in the analytical signal, which should be separately managed and taken into account in the analysis of pesticides, especially in real specimens.

Chemical immobilization in which the recognition element is strongly attached to the support *via* covalent bonds is free from this drawback.[58 – 75] The immobilization is accomplished using various cross-linking agents that react with amino groups (*e.g.*, glutaraldehyde) or carboxyl groups (*e.g.*, carbodiimide), which are present on the surface of the enzyme being immobilized. The enzyme immobilization *via* the formation of disulfide bonds has a very limited practical application. Despite the substantial and persistent stabilization of the catalytic activity remaining in immobilized enzymes after the chemical immobilization, a severe and almost unavoidable drawback of this approach is the considerable loss of the initial enzyme activity upon the action of cross-linking agents. Depending on the particular immobilization conditions, the loss of catalytic activity may vary in the range from 30% to 90%. The considerable inactivation of enzymes during chemical immobilization substantially decreases the economic attractiveness of these recognition elements in enzyme biosensors, and the interest in these works is justified only in the case if the foremost requirement imposed on pesticide analysis is the enzyme stability rather than the level of enzyme activity or the sensitivity of determination.

The listed drawbacks of physical and chemical immobilization are not inherent in the affinity (in particular, metal chelate)[40] immobilization of enzymes.[76 – 81] On the one hand, the interactions arising between the immobilized enzyme and the support are much stronger than in the case of physical immobilization. On the other hand, this type of immobilization does not cause the unrecoverable loss of enzyme activity, like that arising upon the covalent interactions between the enzyme and the cross-linking agent. The affinity immobilization method for the fabrication of an efficiently operating enzyme biosensor is comparable with physical immobilization in the simplicity. For example, comparison of biosensors fabricated from genetically modified AChE (from fruit fly) by physical,[49 – 51] chemical[74] and affinity[79] immobilization methods demonstrated that the manufacturing process lasts for not more than 76, 6 and 0.25 h, respectively, the analytical characteristics of the ready biosensors being similar. At the same time the biosensors have different stabilities (see Table 3).

It should be emphasized, however, that this type of metal chelate immobilization requires preliminary genetic modification of the enzyme, in order to insert a particular amino acid sequence that would specifically react with the functional groups of the support material. This method for the manufacture of an enzymatic recognition element opens up, as a rule, new prospects for the fabrication of biosensors of a particular type with specified characteristics. For example, one and the same enzyme can be genetically modified in such a way that it would strongly and specifically bind to a gold electrode without any additional operations, while for another application model it would bind to magnetic nanoparticles. Actually, this will not be the same enzyme, and the modified analogues can differ in the catalytic characteristics, in particular, in the specificity of action of inhibitors and/or action on the substrate both from one another and from the initial enzyme; hence, the characteristics of biosensors would also be different. Therefore, for success, it is required to carry out interdisciplinary research combining knowledge in the field of analytical chemistry, molecular biology, bioengineering and chemical enzymology.

Diversified organic and inorganic materials can serve as supports for immobilization of enzymes. Currently, composites, including those containing nanomaterials, are used most widely as the supports.[41, 43, 44, 51 – 56, 58 – 60, 62, 64 – 67, 69, 73 – 75] These materials are especially important, as they substantially improve the analytical characteristics of the biosensors based on immobilized enzymes, which is described below.

III.3. Combined enzymatic biosensors based on several enzymes

Combined enzymatic biosensors containing simultaneously enzymes of various nature or variants of the same enzyme can be fabricated by a number of approaches (see Table 3). An example of joint application of enzymes for the determination of pesticides is a multichannel biosensor in which each enzyme generates a separate analytical signal. Since all of the enzyme components are introduced into the biosensor by one and the same method (for example, covalent immobilization on a screen-printed carbon electrode), the subsequent analysis of pesticides is performed by a common procedure, for example, by one-time immersion of the electrode into a sample under conditions that are the same for all components (enzymes) of the biosensor, and the obtained results are compared. The conclusion about the presence of a particular pesticide

Table 3. Examples of biosensors based on immobilized enzymes applied for determination of pesticides.

Enzyme	Immobilization technique	Determination method and limit of detection [a] of pesticides	Residual activity (testing conditions)	Ref.
		Physical immobilization		
MPH (from *E. coli*)	Adsorption on the surface of the Fe_3O_4/AuNPs/SPE composite	Square-wave voltammetry, 0.1 μg litre^{-1} MPT	80% (30-day storage)	41
MPH (from *E. coli*) containing GST polypeptide	Adsorption (absorption) on/in PVDF membrane (Immobilon-P^{SQ})	Colorimetry, 1 mg litre^{-1} MPT (without a colorimeter in real specimens: down to 10 mg litre^{-1}, recovery: 75% – 93%)	80% (84-day storage), 93% (6 measurements)	42
OPH (n.d.) and pyranine conjugate	Adsorption on silica gel-coated AgNPs	Fluorimetry, 1 μg litre^{-1} PX	n.d.	43
His_6-OPH (from *E. coli*)	Adsorption on the RGON film	Amperometry, 37.7 μg litre^{-1} PX	90% (100 measurements)	44
	Inclusion into the Nafion film on the MC/CB/GCE composite surface	Amperometry, 33 μg litre^{-1} PX	n.d.	45
Esterase 2 (from *E. coli*)	Adsorption on a nitrocellulose membrane	Colorimetry, 2.75 mg litre^{-1} PX (in real specimens with the soluble enzyme: down to 2.75 mg litre^{-1}, recovery: n.d.)	100% (60-day storage)	46
Catalase (from bovine liver)	Adsorption on hierarchically porous calcium phosphate on the GCE surface	Amperometry, 3.3 μg litre^{-1} 2,4-D (in real specimens: n.d., recovery: 98% – 103%)	91% (30-day storage)	47
AChE (n.d.)	Commercially available electrode (adsorption on a platinum electrode)	Amperometry, 33.5 μg litre^{-1} CPO (in real specimens: 33.5 μg litre^{-1}, recovery: n.d.)	90% (10 measurements)	48
AChE variants (from fruit fly)	Inclusion in the film of cross-linked polyvinyl alcohol on the surface of carbon SPE	Amperometry, 1.7 ng litre^{-1} CPO, 68 ng litre^{-1} malaoxon (in real specimens, together: down to 33.5 ng litre^{-1}, recovery: 82% – 140%)	100% (20 measurements)	49
	The same	Amperometry, 37 ng litre^{-1} CPO, 0.31 μg litre^{-1} CFV, 64 ng litre^{-1} azinphos-methyl oxon (in real specimens together: down to 30 ng litre^{-1}, recovery: 92% – 111%)	n.d.	50
AChE variants (from fruit fly)	Inclusion in the film of polyvinyl alcohol composite with Fe – Ni nanoparticles on the SPE surface	Amperometry, 32 ng litre^{-1} phosmet (in real samples: down to 32 μg litre^{-1}, recovery: 96%)	100% (30-day storage)	51
AChE (n.d.)	Adsorption on the AuNPs@PDDA/RGO/SPE composite	Amperometry, 27.5 pg litre^{-1} PX	n.d	52
	Adsorption on the PB@CS/AuNPs@βCD/RGO/GCE composite	Amperometry, 4.14 ng litre^{-1} malathion, 1.15 ng litre^{-1} carbaryl	92% (28-day storage)	53
AChE (from human red blood cells)	Adsorption on the AuNRs/GCE or Au@AgNRs/GCE composite	Chronoamperometry, 0.19 μg litre^{-1} PX, 0.89 μg litre^{-1} dimethoate (in real specimens: down to 0.28 mg litre^{-1}, recovery: 96% – 100%)	93% (30-day storage)	54
AChE (from eel)	Inclusion between PAH layers on the PAH@PSS/CdTe QDs composite surface on a glass or quartz support	Fluorimetry, 2.89 ng litre^{-1} PX, 1.3 ng litre^{-1} PT, 0.243 μg litre^{-1} dichlorvos, 12.6 ng litre^{-1} omethoate (in real specimens: down to 2.3 μg litre^{-1}, recovery: 105% – 115%)	100% (35-day storage)	55

Table 3 (continued).

Enzyme	Immobilization technique	Determination method and limit of detection [a] of pesticides	Residual activity (testing conditions)	Ref.
		Physical immobilization		
AChE (from eel)	Adsorption on the PEDOT-MWCNTs/FTO composite	Differential pulse voltammetry, 0.33 pg litre^{-1} malathion (in real specimens: down to 0.33 pg litre^{-1}, recovery: 96 – 98%)	89% (30-day storage)	56
AChE variants (from fruit fly and eel) combined with OPH (n.d.)	Inclusion in a cross-linked polyvinyl alcohol film on the surface of screen-printed carbon electrode	Amperometry, 8.37 ng litre^{-1} CPO, 0.18 μg litre^{-1} CFV (in real specimens together: down to 65 ng litre^{-1}, recovery: 85% – 110%)	n.d.	57
		Chemical immobilization		
MPH (from *Pseudomonas* sp.)	Covalent cross-linking by EDC/NHS with AuNPs on the PEG-coated GCE	Amperometry, 70 ng litre^{-1} MPT (in real specimens: 20 μg litre^{-1}, recovery: 93% – 107%)	90% (70-day storage)	58
	Covalent cross-linking by EDC/NHS with the SP@AuNPs/MWCNTs/GCE composite	Square-wave voltammetry, 0.3 μg litre^{-1} MPT (in real specimens: 1 μg litre^{-1}, recovery: 95% – 102%)	80% (30-day storage)	59
	Covalent cross-linking by EDC/NHS with CdTe quantum dots followed by deposition on the AuNPs/MWCNTs/GCE composite	Amperometry, 5 μg litre^{-1} MPT (in real specimens: 100 μg litre^{-1}, recovery: 96% – 101%)	90% (30-day storage)	60
OPH (from *E. coli*)	Covalent cross-linking by DSC with porous silica gel and coating by PDMAEMA	Effective optical thickness measurement, 9.9 mg litre^{-1} MPX	n.d.	61
	Covalent cross-linking by EDC/NHS with MWCNTs and multilayer deposition on GCE	Amperometry, 21.2 μg litre^{-1} PX	85% (60-day storage), 45% (180-day storage)	62
OPH (from *E. coli*)	Covalent cross-linking by glutaraldehyde with chitosan hydrogel on the GCE surface	Amperometry, 0.55 mg litre^{-1} PX, PT or MPT	50% (1-day storage)	63
	Covalent cross-linking by EDC/NHS with SWCNTs, deposition on GCE and coating by Nafion	Amperometry, 33 μg litre^{-1} PX	75% (210-day storage)	64
Tyrosinase (from fungus)	Covalent cross-linking by glutaraldehyde with ZnONPs/SPE	Amperometry, 0.1 μg litre^{-1} chlortoluron (in real specimens: 10.7 μg litre^{-1}, recovery: 93% – 116%)	n.d.	65
Tyrosinase (n.d.)	Covalent cross-linking by glutaraldehyde with TiO_2NPs/Ti	Amperometry, 0.1 ng litre^{-1} atrazine (in real specimens: down to 1.1 μg kg^{-1}, recovery: 88% – 101%)	90% (28-day storage)	66
Lipase (from *Candida rugosa*)	Covalent cross-linking by glutaraldehyde with PAH/AuNPs/Au	Conductometry, 60 μg litre^{-1} diazinon, 26 μg litre^{-1} MPT, 25 μg litre^{-1} MPX (in real specimens: down to 1.5 mg litre^{-1}, recovery: 67% – 86%)	55% (30-day storage)	67

Table 3 (continued).

Enzyme	Immobilization technique	Determination method and limit of detection [a] of pesticides	Residual activity (testing conditions)	Ref.
		Chemical immobilization		
Paraoxonase 1 (n.d.)	Covalent cross-linking by glutaraldehyde with a 2-mm copper grid for TEM followed by liquid crystal formation	Optical transmission, 0.28 mg litre^{-1} PX	n.d.	68
Alkaline phosphatase (from bovine liver)	Covalent cross-linking with the PVASbQ/MWCNTs/SPE composite	Amperometry, 1 μg litre^{-1} 2,4-D (in real specimens: 25 μg litre^{-1}, recovery: 92% – 100%)	70% (12 measurements)	69
AChE (n.d.)	Covalent cross-linking by EDC/NHS with a gold support on the surface of a piezoelectric AlN oscillator	Change in the resonator oscillation frequency, 11.8 ng litre^{-1} PX	n.d.	70
	Covalent cross-linking by EDC/NHS with a gold support on the surface of a piezoelectric ZnO oscillator	Change in the resonator oscillation frequency, 4.95 ng litre^{-1} PX	86% (15-day storage)	71
AChE (from eel)	Covalent cross-linking with a gold support on the surface of a piezoelectric AlN oscillator by sulfosuccinimidyl 6-[3-(2-pyridylthio)propionamide]-hexanoate	Change in the resonator oscillation frequency, 14.6 ng litre^{-1} PX (in real specimens: 0.5 g litre^{-1}, recovery: 91% – 109%)	91% (10-day storage)	72
	Covalent cross-linking by EDC/NHS with carboxylated chitosan on the surface of the AgNC/RGO/GCE composite	Differential pulse voltammetry, 24.2 ng litre^{-1} phoxim	92% (30-day storage)	73
Variants (from fruit fly and eel)	Covalent cross-linking with a composite nanofibrous chitosan membrane with polyvinyl alcohol on the SPE surface	Amperometry, 61 ng litre^{-1} pirimiphos methyl (in real specimens: down to 9.2 μg litre^{-1}, recovery: 96% – 102%)	90% (42-day storage)	74
AChE (from eel) combined with OPH (from *E. coli*)	Covalent cross-linking by EDC/NHS with MWCNTs followed by layer-by-layer deposition on GCE modified by two layers of MWCNT conjugates with polyethyleneimine or DNA	Amperometry, 0.14 mg litre^{-1} PX, 2 mg litre^{-1} carbaryl	n.d.	75
		Affine immobilization		
His_6-MPH (from *E. coli*)	Binding to NTA-modified and Ni^{2+}-charged agarose	Colorimetry, 1 mg litre^{-1} MPT	n.d.	76
His_6-GBP-OPH (from *E. coli*)	Binding to the gold electrode surface	Chronoamperometry, 10 ng litre^{-1} PX	n.d.	77
	Binding to gold nanoparticles on the surface of reduced graphene oxide	Amperometry, 26.3 μg litre^{-1} PX	n.d.	78
His_6-AChE (from fruit fly)	Binding to IDA-coated magnetic particles charged with Ni^{2+} ions	Amperometry, 14.4 ng litre^{-1} CPO (in real specimens: 100 ng litre^{-1}, recovery: 96% – 103%)	100% (11-day storage)	79

Table 3 (continued).

Enzyme	Immobilization technique	Determination method and limit of detection [a] of pesticides	Residual activity (testing conditions)	Ref.
		Affine immobilization		
His_6-AChE (from fruit fly) combined with OPH (from *E. coli*)	Binding of His_6-AChE to IDA-coated magnetic particles charged with Ni^{2+} ions and covalent immobilization of OPH on sepharose	Amperometry, 4 ng litre^{-1} CPO, 5 ng litre^{-1} CFV (in real specimens: down to 2 μg litre^{-1}, recovery: n.d.)	n.d.	80
His_6-GST-Vmh2 (from *E. coli*)	Binding to the polystyrene microplate surface	Colorimetry, 5 μg litre^{-1} molinate, 1 μg litre^{-1} captan	100% (3-day storage), 80% (50 measurements)	81

Notes. MPH is methyl parathion hydrolase, GST is glutathione-S-transferase, PVDF is polyvinylidene fluoride, AgNPs are silver nanoparticles, AuNPs are gold nanoparticles, Fe_3O_4/AuNP/SPE are AuNP-coated Fe_3O_4 nanoparticles on a screen-printed carbon electrode, His_6-OPH (His_6-MPH, His_6-AChE) is organophosphorus hydrolase (methyl parathion hydrolase, acetylcholinesterase) with His_6 tag genetically attached to the N-terminus, RGON is reduced graphene oxide/Nafion composite, MC/CB/GCE mesoporous carbon and carbon black on a glass carbon electrode, AuNPs@PDDA/RGO/SPE is reduced graphene oxide coated by AuNPs in the presence of poly(diallyldimethylammonium) chloride on a screen-printed carbon electrode, AuNRs/GCE are gold nanorods on a glass carbon electrode, PB@CS/AuNPs@βCD/RGO/GCE is reduced graphene oxide coated by Prussian Blue with chitosan sulfate and AuNPs with β-cyclodextrin on a glass carbon electrode, PAH@PSS/CdTe QDs is a multilayer composite composed of polyallylamine hydrochloride sulfate and polystyrene sulfonate on the surface of CdTe quantum dots, PEDOT-MWCNTs/FTO is poly(3,4-ethylenedioxythiophene) grafted to multiwalled carbon natotubes on a fluorine-doped tin oxide electrode, EDC is 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide, NHS is *N*-hydroxysuccinimide, PEG is polyethylene glycol, DSC is *N*,*N*′-disuccinimidyl carbonate, SP@AuNPs/MWCNTs/GCE are AuNP-coated silica gel submicroparticles on a glass carbon electrode (attachment *via* multiwalled carbon nanotubes), PDMAEMA is poly(2-dimethylaminoethyl methacrylate), SWCNTs are single-walled carbon nanotubes, ZnONPs/SPE are ZnO natoparticles on a screen-printed carbon electrode, TiO_2NPs/Ti are TiO_2 nanoparticles on the Ti surface, PAH/AuNPs/Au are polyallylamine-coated gold nanoparticles on a gold electrode, TEM is transmission electron microscopy, PVA-SbQ/MWCNTs/SPE is styrene-pyridine modified polyvinyl alcohol on a screen-printed electrode coated by multiwalled carbon nanotubes, AgNC/RGO/GCE is reduced graphene oxide coated by silver nanoclusters on GCE, His_6-GBP-OPH is organophosphorus hydrolase with gold-binding peptide and His_6 tag genetically attached to the N-terminus, NTA is nitrilotriacetic acid, IDA is iminodiacetic acid, His_6-GST-Vmh2 is glutathione S-transferase containing His_6-tag at the N-terminus and Vmh2 hydrophobin on the C-terminus.

Detected compounds: CPO is chlorpyrifos oxon [*O*,*O*-diethyl *O*-(3,5,6-trichloropyridin-2-yl) phosphate], malaoxon is *O*,*O*-dimethyl *S*-{1,2-di(ethoxycarbonyl)ethyl} phosphorothioate], CFV is chlorfenvinphos [*O*,*O*-diethyl *O*-{2-chloro-1-(2,4-dichlorophenyl)vinyl} phosphate], azinphos-methyl oxon is *O*,*O*-dimethyl *S*-[(4-oxo-1,2,3-benzotriazine-3(4*H*)-yl)methyl] phosphorothioate, phosmet is *O*,*O*-dimethyl *S*-(*N*-phthalimidomethyl) phosphorodithioate, dimethoate is *O*,*O*-dimethyl *S*-(methylcarbamoylmethyl) phosphorodithioate, omethoate is *O*,*O*-dimethyl *S*-(methylcarbamoylmethyl) phosphorothioate, MPX is methylparaoxon [*O*,*O*-dimethyl *O*-(4-nitrophenyl) phosphate], chlortoluron is 3-(3-chloro-4-methylphenyl)-1,1-dimethylurea, atrazine is 2-(*N*-isopropyl)-6-chloro-4-(*N*-ethyl)-1,3,5-triazine-2,4-diamine, phoxim is *O*,*O*-diethyl *O*-(α-cyanobenzylideneamino) phosphorothioate, pirimiphos methyl is *O*,*O*-dimethyl *O*-(2-diethylamino-6-methylpyrimidin-4-ol) phosphorothioate), molinate is *N*,*N*-hexamethylene-8-ethylthiocarbamate, captan is 1,2,5,6-tetrahydro-*N*-trichloromethylthiophthalimide.

[a] In the testing of real specimens, the limit of detection or the minimum concentration of pesticides subjected to analysis and the recovery referred to that for the reference method (HPLC, GC/MS, *etc*.) are given.

is drawn on the basis of different specificities of enzymes towards the same substrate or inhibitor.[49 – 51, 74]

One more way of joint application of enzymes for the determination of pesticides is an additive biosensor in which one component (enzyme) is present simultaneously in several parts of the biosensor or analytical cell, and the other component is added to it as necessary (most often, in the soluble form).[57, 80] A modified approach to the fabrication of enzymatic biosensors is the simultaneous immobilization of various enzymes in one biosensor.[75] The most dramatic example of implementation of this method is a system of consecutive (conjugated) reactions, for example: analyte → product-1 → product-2 and so on.[39] As a result, it becomes possible to considerably extend the range of enzymes that can be utilized in the fabrication of a biosensor. Furthermore, this approach often allows one to avoid the effect of compounds and/or conditions interfering with the determination, especially for detection of pesticides in real specimens. However, this requires targeted optimization of the component ratio for improving the reproducibility of the analytical signal and other characteristics of the enzymatic biosensor (storage stability, sensitivity and so on).

Analysis of the currently designed systems (see Table 3) indicates that, irrespective of the approach used to fabricate the multi-enzyme biosensor, any of the above-indicated techniques can be used for immobilization. The key factor is the applicability of a particular immobilization procedure for all components of the combined system. For the manufacture and application of multi-enzyme biosensor systems, it is especially important to thoroughly choose the conditions of analysis. Usually compromise conditions are required to be suitable for all the enzymes used (pH, ionic strength, buffer capacity, detectable concentration range and so on).

Combining of various enzymes in one biosensor system often not only integrates the properties of particular enzymes into a common analytical system, but also provides analytical characteristics inaccessible with these components used separately.

III.4. Enzymes as parts of living cells

Microbial cells have a number of obvious advantages over enzymes for the use as recognition elements in biosensor systems, because they can metabollically respond to various substances. Quite a few studies are concerned with the development and characteristics of biosensors based on microbial living cells and used for determination of pesticides in various objects. The determination of pesticides in these systems is based on metabolic, respiratory, luminescent and enzymatic activities of cells; however, in this review, we distinguish the microbial biosensors that contain enzymes for determination of pesticides.[82–91] In this case, there is, most often, no need in the expensive procedure of isolation of enzymes located inside the cells. Hence, enzymes occur in the native conformation and, therefore, they are active and stable with time. Not only living cells, but also non-living cells that retain their integrity and native intracellular localization of the enzyme are suitable as recognition elements for such analytical systems.

Despite the simplicity of application of biosensors with enzymes located within whole cells, these biosensors for pesticides are characterized, most often, by diffusion restrictions to mass exchange processes in which pesticides participate as enzyme substrates or inhibitors. Therefore, the sensitivity of biosensors of this type is markedly lower than that inherent in biosensors based on highly purified and immobilized enzymes (see Section IV.3 and Table 3).

The catalytic activity of enzymes within whole cells can be enhanced by using recombinant microorganisms with increased level of synthesis of the enzymes that play a key role in the pesticide determination; most often, these are hydrolytic enzymes (methyl parathion hydrolase, organophosphorus hydrolase and so on).[82–91] The enzyme location in the cell (cell wall, periplasm, cytoplasm) after expression of the corresponding genes can be predetermined by genetic engineering methods by means of the designed DNA constructs based on the appropriate plasmids. First, the maximum proximity of enzymes to the cell surface[84–85, 88] increases the effective catalytic activity of enzymes by reducing the restrictions to mass exchange and increasing the sensitivity of pesticide determination. Second, in this case, the concentration of the enzyme synthesized in the periplasm (enzyme localization of up to 35% of the whole amount in the cell)[87] or on the cell surface (enzyme localization of up to 80%–90% of the whole amount in the cell)[84] will not exceed 1%–3% of the sum of all cell proteins; hence, this method for increasing the catalytic activity level and the reactivity of the cells has a pre-determined limit for the possible improvement.

In the case of synthesis of a recombinant enzyme in the cell cytoplasm (enzyme localization of down to 90% of the whole amount in the cell),[87] the local concentration of the target enzyme markedly increases; however, transmembrane processes form the key obstacle in the enzymatic determination of pesticides. This obstacle is overcome, as a rule, by increasing the cell wall permeability using special chemical permeabilization agents, but this is, in turn, accompanied by increasing danger of loss of cell integrity upon any mechanical impact (for example, stirring, washing with buffer solutions and so on) and considerable deterioration of characteristics of the recognition element. In order to avoid the disruption of the integrity of partly permebealized cells, the cells are treated with various cross-linking agents, coated by gel structures or encapsulated into polymer supports with successful combination of immobilization methods.[82, 83, 89]

IV. Application of enzymes and biosensors based on them for the determination of pesticides

Currently, the transduction of the response of enzyme-based sensing elements into a signal that can be recorded and interpreted is carried out by a variety of methods: optical (including classic methods: spectrophotometry, fluorimetry and chemiluminometry; and more advanced methods: fluorescent quantum dots, surface plasmon resonance), electrochemical (namely, amperometry, potentiometry and conductometry; cyclic voltammetry; impedance determination), micromechanical (for example, piezoelectric resonators, surface acoustic resonance and microcantilevers[92]). Combinations of various enzymes and recording systems provide biosensors with the desired sensitivity, productivity and cost. The last-mentioned issue is especially important for wide spreading and practical application, because this characteristics is often demonstrated by researchers as a reason for feasibility of using biosensors for large-scale monitoring of pesticides, for example, in environmental objects, agricultural products, *etc*. The achievements related to practical implementation of enzymatic biosensors most interesting from theoretical and applied standpoints are considered below.

IV.1. Soluble enzymes applied for determination of pesticides

Historically, soluble enzymes used for pesticide determination appeared in the early days of bioanalytical chemistry; their development was largely promoted by expansion of scientific knowledge, first of all, in biocatalysis and structural chemistry of proteins. Modern designs of such analytical systems imply the use of diversified methods of signal detection. Optical methods are preferable because of their simplicity, while the fluorescence method is most sensitive (see Table 2).

Although the use of fluorescent substrates, or substrates that give rise to fluorescent products in reaction systems upon the action of enzymes, or recognition elements (enzymes) containing chemically or genetically introduced fluorescent labels has already become traditional in modern analytical systems, nanoobjects, *e.g.*, quantum dots, also become more and more popular as constituents of bioanalytical systems.[25–26, 33–34, 38] This approach allows a considerable increase in the sensitivity of pesticide determination in real specimens (by 2–3 orders of magnitude, down to ng litre^{-1})[33–34] as compared with analogous systems without quantum dots. This sensitivity level is already sufficient to meet

the established maximum permissible levels of pesticides in test specimens (see Table 1) and to successfully compete with other, non-enzyme analytical methods utilized to determine pesticides.

It is noteworthy that most of the considered bioanalytical systems do not show selectivity between classes of pesticides and, to a large extent, also within the classes. Therefore, it is necessary to develop special procedures (sample preparation, analysis design and so on) for dealing with this drawback (see Section III.3). Nevertheless, even now it is already possible to apply such enzymes for fast *in situ* testing of samples for the general or group detection of pesticides (Table 4).

The electrochemical method for determination of pesticides using soluble enzyme forms as recognition elements [30, 39] appears less attractive from the practical standpoint than optical methods, in particular, fluorescence-based ones. Because of the relatively low sensitivity and procedural complexity, it appears unlikely that test kits would be developed; therefore, this area of research using soluble forms of enzymes is barely addressed now. However, the situation with biosensors based on immobilized enzymes is, in principle, different; in this case, electrochemical methods of determination of pesticides provide more practically useful results.

IV.2. Biosensors based on immobilized enzymes

Biosensors based on immobilized enzymes imply the use of a broad variety of detection methods; however, optical and electrochemical methods are most popular (see Table 3). These two types of methods possess comparable sensitivity of pesticide analysis in model systems (limit of detection of down to ng litre^{-1}). Electrochemical methods provide considerable benefit in the analysis of real specimens (down to ng litre^{-1}). Nevertheless, both detection methods fully correspond to the demands of practice (see Table 1), the choice of a particular method for determination of pesticides being dictated by the analytical task to be solved.

Comparable limits of detection of pesticides are apparently due to the extensive use of the same nanoobjects; furthermore, biosensors with immobilized enzymes, unlike those with soluble enzymes, can employ not only quantum dots, but also nanoparticles, nanotubes and their combinations. On the one hand, this substantially (by a factor of five and more) enhances the analytical characteristics of the enzymatic biosensor [78] and, on the other hand, provides new opportunities for pesticide detection, for example, by utilizing surface plasmon resonance [77] on a gold electrode for analytical purposes. One more example is the use of magnetic nanoparticles in a flow system [79] in which the enzymatic biosensor is separated from the reaction medium containing the analyte by applying a magnetic field. It is worth noting that optimization of both the quantitative and qualitative composition of these enzymatic nanosensors is required in each case. For example, it was shown that single-walled nanotubes provide better catalytic and analytical characteristics of the biosensor based on the organophosphorus hydrolase than multiwalled nanotubes [64] for determination of organophosphorus pesticides. In this respect, biosensors obtained by metal-chelate immobilization of enzymes on deliberately prepared supports are quite promising and interesting. With this method of enzyme immobilization, biosensors can be specially designed for solving particular analytical tasks. For example, a

Table 4. Examples of commercially available enzyme-based systems for determination of pesticides with optical signal recording.[a]

Name	Manufacturer	Limit of detection	Website
aCella-AChE assay kit	Cell Technology	1.6 mg litre^{-1} malathion	www.celltechnology.com
CideLite	Charm Sciences	0.3 μg litre^{-1} chlorpyrifos, 0.1 μg litre^{-1} coumaphos, 10 μg litre^{-1} malathion, 0.1 μg litre^{-1} MPT [b]	www.charm.com
MAIA Pesticide MultiTest	Liofilchem	10 μg kg^{-1} PX, 100 μg kg^{-1} carbaryl, 100 μg kg^{-1} DDT (in real specimens)	www.liofilchem.net
OP/Carbamate Assay	Abraxis	0.5 μg litre^{-1} chlorpyrifos, 1.2 μg litre^{-1} malathion, 0.6 μg litre^{-1} diazinon [b]	www.abraxiskits.com
Eclox Rapid Response Test Kit [c]	Hach	—	www.hach.com
Pesticide Detection Cards	RenekaBio	2 mg litre^{-1} malathion, 2.5 mg litre^{-1} carbaryl, 1.7 mg litre^{-1} PT [b]	www.renekabio.com
NIDS ® ACE III-C	ANP Technologies	2 mg litre^{-1} fenamifos, 2 mg litre^{-1} oxamyl	www.anptinc.com
Pesticide In Water test kit	PRO-LAB	3 μg litre^{-1} atrazine, 4 μg litre^{-1} simazine	www.prolabinc.com
Acetylcholinesterase BIOSENSOR AC1.AChE [d]	BVT Technologies	—	www.bvt.cz

[a] All specimens use various forms of AChE as the base. [b] Only some of the characteristics are given. Analogous parameters for the other compounds can be found in the reagent kit manual or the manufacturer website. [c] The kit includes various test systems, in particular, for determination of pesticides. [d] Electrochemical signal recording is used.

biosensor based on genetically modified organophosphorus hydrolase and using highly sensitive surface plasmon resonance method would determine an analyte in concentrations down to 10 ng litre^{-1} (Ref. 77). Therefore, one should expect that the number of publications describing pesticide determination with limits of detection of an order of pg litre^{-1} (Ref. 52) may markedly increase in the near future.

Here, it is necessary to note that the possible causes for the sharp improvement of the limits of detection (by 2–3 orders of magnitude) for biosensors using enzyme inhibition and nanomaterials are actively discussed in the scientific community. There is a hypothesis that inhibitors are concentrated in an immobilized enzyme layer. Nevertheless, this improvement may be caused by other reasons; therefore, additional investigations are required.

IV.3. Biosensors based on combinations of several enzymes and enzymes in the cells

Combination of various enzymes in one biosensor system often not only adds the properties of components, but also provides characteristics inaccessible for these components taken separately. Therefore, it is quite reasonable and justified to combine cholinesterases, for example, with enzymes that hydrolyze the analyte (see Table 3).[57,75,80] With this approach, the determination of pesticides in the analyte is performed in parallel along two paths, one of which involves additionally introduced hydrolase. Thus, it is possible to perform discrimination analysis between classes of pesticides using one system (for example, between organophosphorus pesticides and carbamates), because hydrolase acts only on one class of compounds (in this case, organophosphorus pesticides), thus decreasing the cholinesterase inhibition.

The invoking of artificial neural networks and chemometrics makes it possible to discriminate between test compounds even within one and the same class of pesticides.[93] Chemometric analysis of this type can also be carried out using mutant forms of the same enzyme. Mwila *et al.*[94] utilized different genetic modifications of one enzyme — acetylcholinesterase. The strategy of this enzymatic analysis is that during determination, the analyte containing various pesticides is brought in contact with each recombinant enzyme differing from its analogues in catalytic characteristics and, in particular, in the specificity of action of inhibitors. This forms a mathematical matrix composed of analytical signals detected upon the use of different mutant enzymes treated with various pesticide mixtures. Then artificial neural networks can be trained[95] to be applied for interpretation of experimental data obtained for samples with unknown composition.[49]

Biosensors based on cells as the source of enzymes are suitable for determination of paraoxon, parathion and methyl parathion in the concentration range of 0.13 to 175 mg litre^{-1} with optical and electrochemical signal recording.[83–86]

Whole-cell enzymatic biosensors can be formed on the basis of screen-printed carbon electrodes,[82,89] which enables repeated use of these biosensors (up to 40 operation cycles) with not more than 20% decrease in the initial characteristics for the indicated range of pesticide determination.[82]

Also, there are biosensors based on whole cells that contain not only the enzyme directly participating in the reactions with pesticides, but also a protein, in particular, green fluorescent protein,[87] co-expression of which with the target enzyme is genetically programmed, for example, on the cell surface and the fluorescence level of which depends on the concentration of accumulated products of pesticide destruction. These enzymatic biosensors as whole cells that express different proteins extend the range of eligible pesticides, in particular, it is possible to detect fenitrothion, diazinon and chlorpyrifos and not only those compounds that undergo enzymatic hydrolysis to give a light-absorbing product, for example, *p*-nitrophenol. However, the concentrations that can be determined by such biosensors are still insufficient for practical application (8.7 and 10 mg litre^{-1} for parathion and methyl parathion, respectively).[87]

V. Conclusion

Today, enzymatic biosensors have passed a long way from the first designed items to commercial products (see Table 4). These biosensors can be combined with various methods of analytical signal recording; they can contain various enzymes in either soluble or immobilized form and so on — all these characteristics are related to the particular analytical problem to be solved. Most of the known designed systems fully comply in their sensitivity with the modern requirements to the permissible pesticide levels in various specimens (see Table 1) and are also very simple in application for consumers.

Nevertheless, this does not eliminate the drive of researchers to further improve the limits of detection of compounds by enzymatic biosensors. The application of a variety of nanoobjects in combination with advanced methods for detection of pesticides can markedly improve these characteristics. In our opinion, a breakthrough based on the use of nanomechanical detectors and signal transducers can be expected in the near future.[92] A similar breakthrough was provided five years ago by the development of the reagentless optical detection of AChE inhibitors. For example, Febbraio *et al.*[46] determined esterase activity by adapting an approach used previously only in enzyme immunoassay. The approach is as follows: an enzyme (in this particular case, esterase 2 from *Alicyclobacillus acidocaldarius*) is immobilized on a nitrocellulose membrane *via* physical adsorption, then the enzyme is brought in contact with the pesticide being determined and then the enzyme substrate is added; the product of substrate hydrolysis develops a colour in the presence of a 'witness' (Fast Blue RR). The colour intensity is used to detect the inhibitor and estimate its concentration by naked eye. Further development of this design comprising immobilization of the enzyme together with the substrate and the witness, which then move and are mixed by the front of the analyte, served as the basis for fabrication of numerous commercially available test strips for rapid and simple assay of pesticides (see Table 4).

One more promising approach to the determination of pesticides that may form in the near future is obviously the use of surface-enhanced Raman spectroscopy,[96] which is often more sensitive than other types of surface plasmon resonance and can detect various compounds directly in the specimen (without sample preparation). This method is also applicable for pesticide analysis using gold or silver nanoparticles, in particular, with the use of enzymes.[97]

A considerable potential is inherent in flow type and microfluidic enzymatic biosensors. Only a few studies have now been devoted to the development of analytical systems of this type. Nevertheless, most recent publications[98] attest to the possibility of successful adaptation of enzymatic biosensors to classic chromatographic determination methods; therefore, the number of studies devoted to this topic should be expected to increase.

Yet another trend for the further development of enzymatic biosensors is determination of new types of pesticides. Indeed, the enzymatic biosensors known to date are designed for determination of 'classic' pesticides, that is, traditionally used pesticides and none of the biosensors is suitable for determination of modern 'biopesticides'. These include neonicotinoids and insect growth regulators and, as shown by recent investigations, they are far from being absolutely safe, as it was suggested earlier.[99] For example, in 2013, the European Union introduced a two-year moratorium (http://europa.eu/rapid/press-release_IP-13-379_en.htm) on the use of three neonicotinoids (clothianidin, imidacloprid and thiametoxam) because of their high hazard for honey bees, and France even extended this ban to 2018. Therefore, more stringent regulations of the use of these compounds and monitoring of their presence in the environment may appear in the near future. Since all biopesticides have natural origin and are not xenobiotics, *i.e.*, they must be susceptible to the action of some enzymes, they all, in principle, can be detected by using enzymatic biosensors based on new enzymes active in the reactions with newly introduced biopesticides.[100]

It should be emphasized that the practical use of enzymatic methods to monitor the presence of pesticides in environmental objects is still limited. Among these objects, especially important are water and soil resources; raw materials and agricultural and/or food industry products; consumer goods and so on. Apart from the lack of the regulatory base needed for the application of enzymatic biosensors, restrictions are related to the stability of the analytical signal, in particular, on their storage. This problem can be solved by developing new, 'mild' methods for stabilization of enzymes in biosensors, for example, by forming stable non-covalent enzyme – polyelectrolyte complexes. Genetic engineering techniques can obviously help in increasing the selectivity of enzymatic determination of pesticides functioning as enzyme inhibitors, first of all, in multicomponent media. Specially developed multienzyme systems that can discriminate between different pesticides are the most promising ones.

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