APPLICATION OF GEL SYSTEMS WITH VARIOUS BIOCATALYSTS DETOXIFYING NEUROTOXIC AGENTS FOR POLLUTION CONTROL, WATER PURIFICATION, AND SELF-DEFENSE

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Abstract. The development and potential application of various biosystems based on enzymes and living cells of microorganisms, immobilized into gel-containing carriers and capable of detoxifying neurotoxic agents such as organophosphorous pesticides and chemical warfare agents as well as products of their destruction are discussed.

Keywords: Immobilized biocatalysts, organophosphorus hydrolase, polyhistidine sequence, organophosphorous pesticides, chemical warfare agents, poly(vinyl alcohol) cryogel, cross-linked sulphate chitosan gel, polyacrylamide cryogel.

1. Introduction

The organophosphorous pesticides are extensively applied in agriculture despite their mammalian neurotoxicity. The world market analysts gave the following prognosis: the e turnover of the market sector will grow per 11.7% annually and reach the $210 billion up to 2007 year. The abundant application of organophosphate compounds (OPC) along with their low water solubility results in the accumulation of the compounds in soil, ground and river waters. All OPC possess acute or delayed neurotoxicity, but the cumulative mutagenic effect of low OPC quantities appeared to be even more dangerous than neurotoxic one. The comprehension of real OPC threat for human health starts to be absolutely clear when someone take into account that OPC penetrate into the human body

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via skin, inhalation and with water and foods. Today, the costumers widely used the OPC as domestic insecticides to treat their farmlands, backyards and pets without appropriate control compose the largest group of people who risk their health every day.[1] The diagnosis of cancerous or chronic diseases as result of OPC poisoning became reality of our days especially for those who participates in the production of pesticides or works in contact with OPC.[2,3]

According to Chemical Weapons Convention, 1,000 t of stored organophosphorous warfare agents (Vx, sarin, soman) should be destroyed in various countries during next several years. The technologies accepted for the destruction of chemical warfare agents (CWA) have various disadvantages (non-destructed residual amounts of toxic CWA, corrosive or flammable wastes, etc.) and all means enabling their elimination are of high scientific and practical importance.

Thus, the development of effective, user-friendly and economically appropriate biocatalytic systems that can prevent or reduce the threat of toxic influence of OPC used for the indoor and agricultural purposes as well as stored in depots is one of the main tasks of up-to-date biotechnology.

Organophosphorus hydrolase (OPH, EC 3.1.8.1) being a key enzyme in the detoxification of mentioned compounds catalyses the hydrolysis of a rather broad spectrum of OP neurotoxins and can be used for "Green solution" of the problem.[4]

The immobilization of OPH and whole cells containing OPH into gel systems appeared to be the most effective ways in the obtaining of biocatalysts with high catalytic activity, long shelf life and half-life time in the operating systems that could be used for pollution control, water purification, and self-defense. The examples illustrating this point of view are present in this review.

2. Gel Biocatalysts for Pollution Control in the Processes of Detoxification of Neurotoxic Organophosphorous Compounds

2.1. ENZYMATIC BIOCATALYSTS IN THE GEL-CONTAINING CARRIERS FOR CONTROL OF DETOXIFICATION OF NEUROTOXINES

The use of the enzyme as an active part of biosensors for discriminative OPC determination stimulates the development of its forms stabilized via immobilization in gel systems.[5,6] The attachment of OPH to various gel carriers (silica gel, poly(vinyl alcohol cryogel)) via multiple covalent bounds,[5,7] was successfully applied in the development of biocatalysts for detection of various OPC concentrations.

The direct control of efficiency of OPC detoxification was realized owing to combination of OPC hydrolysis with accumulation of hydrolytic products with acidic pH values. So, potentiometric analysis was accomplished by using a flow-through bioassay system with Corning glass pH-microelectrode.

Owing to immobilization, the stability of OPH in the solutions with acidic pH values was considerably higher as compared to its free form. The covalent immobilization of OPH[8] being a part of non-covalent complex, formed by enzyme with 1,5-dimethyl-1,5-diazaundecamethyene polymethobromide (polybrene), provided five-fold increase in stability of biocatalyst in 15% (v/v) ethanol solution as compared to soluble enzyme.[9] Immobilized biocatalyst, prepared with an addition of polybrene, retained a half of its initial activity in 50% (v/v) aqueous ethanol solution, 90% of activity during 10 working cycles at pH 6.5–7.5, when the optimum of enzyme action was 8.0–9.0.

The discriminative detection of OPC in various mixtures with carbamates was demonstrated, when combined biosensor containing both immobilized OPH and acetylcholinesterase was used. The detection ranges of this integrated biosensor was 10^{-9} to 10^{-5} M for paraoxon or diisopropyl fluorophosphate. The elimination of organophosphorous neurotoxins from different multi-component inhibitor combinations via sample pretreatment with immobilized forms of OPH was shown to be possible. Since then, the inhibiting influence of non-OPC on the acetylcholinesterase could be separated and its true value might be determined by this approach.

2.2. TEST-SYSTEMS BASED ON APPLICATION OF LIVING CELLS

A biosensor for direct detection of OPC was developed using recombinant *Escherichia coli* cells with OPH activity, immobilized into cryogel of poly (vinyl alcohol).[10] The same potentiometric approach to determination of OPC concentrations as in case of immobilized OPH was utilized here. The linear detection range for paraoxon detection was 0.25–250 ppm (0.001–1 mM), the response times were 10 and 20 min in the batch and flow-through reactors, respectively. It was possible to use same sample of immobilized biocatalyst repetitively for 25 analyses with a 10 min intermediate washing of granules with cells, required for reestablishing starting conditions.

Recently, another one immobilized biocatalyst for the detection of various OPC was developed on the basis of *Photobacterium phosphoreum* cells. It is known, that photobacteria are commonly used test-biosystem allowing revealing of various types of ecological toxins.[11] However, their use in suspended state is limited by several disadvantages: low enough stability of biolumine-scent signal

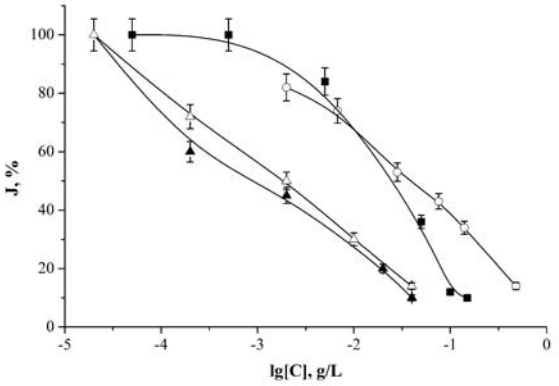


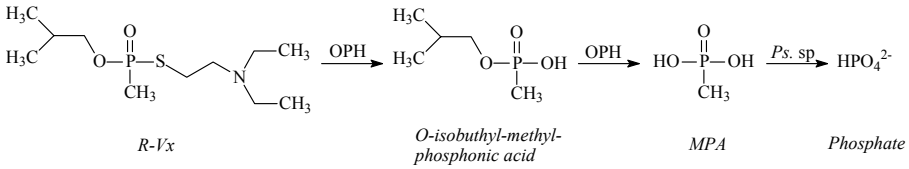
Figure 1. Decrease in bioluminescence of immobilized photobacteria under the inhibiting action of following organophosphorous compounds: ● – methylphosphonic acid, ▲ – malathion, ■ – coumaphos, △ – parathion.

at temperatures over 28°C, storage only in lyophilized form, etc. The cryogel of poly(vinyl alcohol) (PVA)[12] was used as a carrier for photo-bacteria in our work.

It was shown that immobilization of cells did not decrease the light emission and considerably stabilized the level of registered bioluminescence. The storage time of immobilized cells without reduction of initial level of luminescence was 1 month at –20°C and 6 month (as minimum) at –80°C. The concentrations of various compounds inhibiting bioluminescence of immobilized photobacteria were established (Figure 1).

The ranges of concentrations detected for various OPC by immobilized cells were following: 5–150 ppm coumaphos, 0.02–6 ppm parathion, 0.02–40 ppm malathion. The possible determination of concentrations of methylphosphonic acid (MPA) being product of hydrolysis of all known organophosphorous chemical warfare agents (scheme 1) was demonstrated for immobilized photobacteria for the first time. The range of detected concentrations for MPA was 2–500 ppm.

Thus, it was established, that it is possible to use the photobacteria immobilized into PVA cryogel for quantitative determination of neurotoxins.



Scheme 1. Biological hydrolysis of R-Vx.

3. Biocatalysts Immobilized in Gel-Containing Carriers for Detoxification of Neurotoxic Organophosphorous Compounds

3.1. BIOCATALYSTS BASED ON IMMOBILIZED LIVING CELLS-PRODUCERS OF ENZYMES CATALYSING OPC HYDROLYSIS

Recently, a new line of enzymes decontaminating various toxins was developed by Genencor Int. in partnership with U.S. Army's Edgewood Chemical Biological Center (Table 1). Genencor plans to spread these new products selling them to military and civilian fire departments and hazardous material team as well as to apply the products for the bioremediation of industrial and agricultural waste sites. The stabilized enzymatic preparations with general name DEFENZ can be used in immobilized state being introduced into the content of the fire foam.[13]

TABLE 1. Commercial products for OPC destruction.

Company/product	Objects for treatment	References
Genencor Int.		
DEFENZ^{TM} 120 BG	Flat surfaces,	www.genencor.com
DEFENZ^{TM} 130 BG	ground	
Reactive Surfaces, Ltd.	Paints,	www.reactivesurfaces.com
OPDtox^{TM}	coatings	

Another one commercial product (OPDtox^{TM}) for OPC detoxification was developed by Reactive Surfaces, Ltd. as an additive for various environmentally friendly paints and latex coatings. This additive contains a dried whole-cell powder of *E. coli* DH5α cells with OPH activity.[14]

Thus, the commercial immobilized biocatalysts based on enzyme and whole cells with detoxifying activity against neurotoxic organophosphorous compounds are already known. Nevertheless, new variants of effective biocatalysts for OPC destruction are continued to be developed.

It was established that immobilization of whole cells capable of degrading OPC into porous structure of poly(vinyl alcohol) cryogel guaranteed the simple biotechnological procedure and a high viability of entrapped cells during their long-term use for OPC detoxification[15,16] as well as long-term storage.

3.2. BIOCATALYSTS BASED ON IMMOBILIZED ENZYMES WITH ACTIVITY OF ORGANOPHOSPHORUS HYDROLASE

To develop effective biocatalysts with OPH-activity suitable for flow systems, various approaches to enzyme immobilization have been applied.[5,15] The use of

various amino acid sequences genetically introduced into OPH structure as ligands for enzyme immobilization appeared to be advanced and very effective method for biocatalyst production.[15] The use of specific amino acid sequences allowed to combine the isolation, purification and immobilization of the target protein in one step.[17]

Macroporous hydrogel, so called cryogel produced by cross-linking radical polymerization of acrylamide[18] and modified by metal-chelating ligands charged by divalent metal ions, was used as a carrier. Polymer structuring or polymerization of monomers at sub-zero temperatures results in the formation of cryogels in solidified state. The freezing of the solutions of polymers or monomers with the addition of cross-linking agents allows to synthesize the monolith with three-dimensional structure containing numerous pores of 10–100 μm size. At the same time the carrier is the integral monolith allowing to get a notable decrease in diffusional limitations when it is used in a column reactor. The large size of pores allows for liquid to flow through the cryogel monolith with high enough flow rates while sizeable pore surface provides a sufficient area for its modification by metal-chelating ligands and further effective enzyme immobilization.[17] The large size of pores in the cryogel structure enables the use of cell extracts containing the target proteins for their direct loading onto the carrier without additional purification steps and with no risk of blocking the pores by cell debris.

New immobilized biocatalysts based on polypeptides containing polyhistidine (polyHis-) sequences at the N- or C-terminus of protein molecules and possessing activity of organophosphorus hydrolase were developed[19] and investigated for detoxification of organophosphorous neurotoxic compounds in the flow systems.[15,20,21,22]

The catalytic characteristics of the biocatalysts (catalytic constants, temperature and pH-optima) were very similar to each other, independently on genetic modification of OPH used for their production. The biocatalysts had high enough usage and storage stability. It was established that tried biocatalysts could be multiply regenerated by elution of inactive enzyme from the carrier with chelating agent and repeatedly loaded by active polyHis-tagged OPH. It was possible to carry out 10–15 reloading procedure.

The influence of the carrier composition on the catalytic characteristics of the immobilized biocatalysts was studied. Modification of the carrier was carried out via variations of co-monomer groups (allyl glycidyl ether) containing residue of iminodiacetic acid. The increase in ether portion guaranteed increase in amount of polyHis-tagged OPH bonded with the carrier (Figure 2). At the same time, there was no significant difference in the catalytic characteristics between biocatalysts based on carriers with different chemical content (Figure 3).

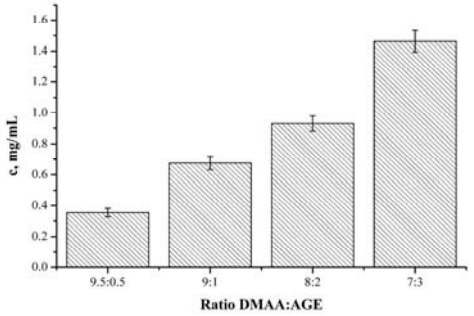


Figure 2. Influence of the monomer composition on the protein concentration immobilized onto the carrier. DMAA – *N,N'*-dimethyl-acrylamide, AGE – allyl glycidyl ether.

It was shown that some samples of gel-based biocatalysts could be dryed-reswollen with more than 90%-retaining of initial enzyme activity. These biocatalysts with immobilized OPH derivatives were successfully tried for OPC detoxification in the flow water systems. It was revealed, that volume of samples of immobilized biocatalysts can be varied from 5 up to 50 ml with retention of all catalytic characteristics. The regular velocity of flow of OPC solutions passed through the samples of immobilized biocatalysts was close to 120 ml/h.

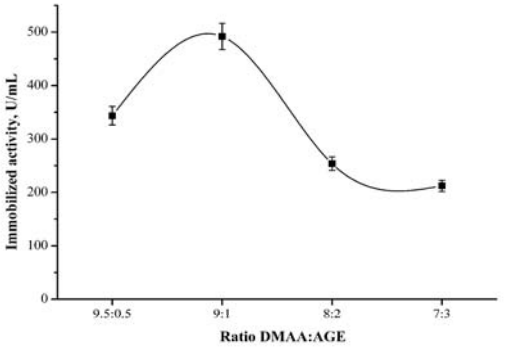


Figure 3. The dependence of specific activity of immobilized biocatalyst with His_6-OPH on the monomer composition: DMAA – *N,N'*-dimethyl-acrylamide, AGE – allyl glycidyl ether.

3.3. CELLS OF MICROORGANISMS IMMOBILIZED INTO CRYOGEL OF POLY(VINYL ALCOGOL) FOR DESTRUCTION OF HYDROLYTIC PRODUCTS OF ORGANOPHOSPHOROUS NEUROTOXINS

The reactions of degradation of organophosphorous chemical warfare agents usually results in accumulation of certain neurotoxic ethers of MPA (O-isopropyl-methylphosphonate, O-isobutyl-methylphosphonate, O-pinacolyl-methylphosphonate).[23] The further destruction of MPA is a significant problem

since C-P bond possesses high resistance to chemical hydrolysis. The disruption of C-P bond present in the chemical structures of three main organo-phosphorous chemical warfare agents (Vx, sarin, soman) is considered as irreversible conversion of the substances (Scheme 1). Until the bond is not destructed it is possible to use the MPA as precursor for restored synthesis of the neurotoxins. It is know, that only microbial conversion of MPA is effective, since microbial cells needed in source of phosphorus in nutrition media could catalyze the disruption of C-P bond.

Recently, new cryogel PVA-immobilized biocatalyst was developed on the basis of *Pseudomonas sp.* capable of destructing MPA.[24] It was revealed, that immobilized cells did not need in long-term adaptation to the toxic substrates like MPA or its ethers.

The immobilized cells were characterized by considerably improved stability to high concentrations of MPA (up to 0.5 g/l) as compared to suspended bacterial cells. The multiple applications of immobilized cells *Pseudomonas sp.* in the process of MPA degradation was demonstrated (Figure 4). The velocity of MPA destruction under batch conditions was actually the same in several working cycles of immobilized biocatalyst (6.6 mg/l/h or 158 mg MPA per one working cycle).

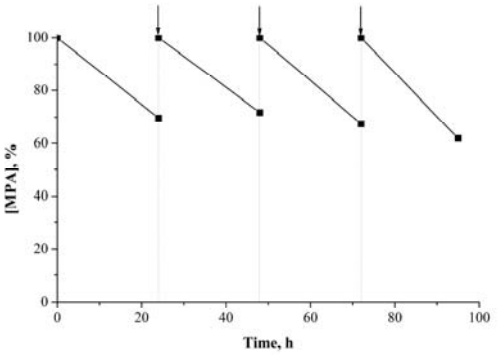


Figure 4. The degradation of methylphosphonic acid in cultural medium during multiple use of immobilized biocatalyst based on *Pseudomonas sp*. Arrows show the replacement of spent cultural medium by fresh one.

The possible reutilization of biocatalyst for MPA degradation was demonstrated (Table 2, Figure 5). To reveal that, the granules of immobilized biocatalyst used for 70 h in the process of MPA degradation were washed with tap water and thawed at 108°C for 20 min.

The obtained PVA solution contained residual concentrations of protein being a part of cell debris. The solution was used to form new portion of granules with immobilized cells. The analysis of mechanical and catalytic characteristics of fresh and reutilized biocatalysts revealed their similarity.

TABLE 2. Characteristics of initial and reutilized samples of immobilized biocatalyst based on *Pseudomonas sp.* cells and PVA cryogel.

Characteristics	Immobilized biocatalyst	
	Initial sample	Reutilized sample
ATP, mol/g biocatalyst	2.01×10^{-9}	1.01×10^{-9}
Protein, mg/g biocatalyst	2.0	4.3
Modulus of elasticity, kPa	65.3 ± 3.8	62.8 ± 4.1

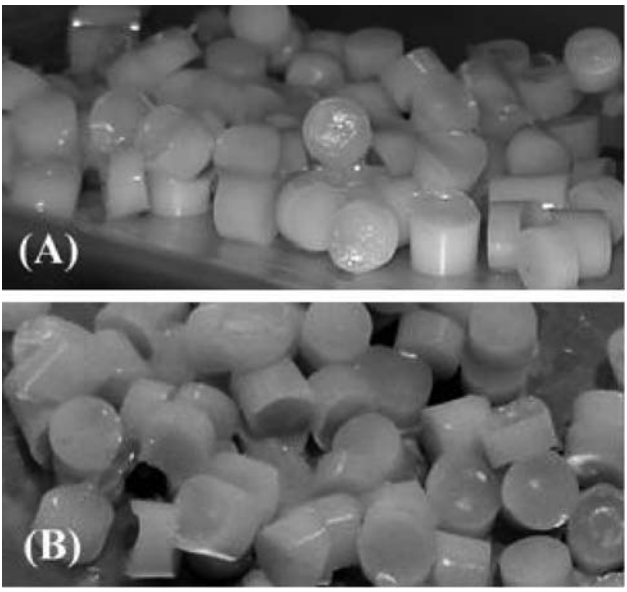


Figure 5. The view of granules of immobilized biocatalyst with *Pseudomonas sp.* cells, immobilized into PVA cryogel: A – initial sample, B – reutilized sample.

TABLE 3. Modulus of elasticity of granules of immobilized biocatalyst formed during the growth of filamentous *Aspergillus niger* cells.

Time (h)	0	24	48	72	96
Modulus of elasticity, kPa	56.5 ± 3.1	68.7 ± 5.3	79.6 ± 8.0	79.6 ± 8.0	78.6 ± 3.2

Another one biocatalyst was recently developed for the degradation of such a product of chlorpyrifos degradation as 3,5,6-trichloro-2-pyridinol (TCP). This compound possesses the toxic properties of both chloride- and organophosphorous compounds and should be destructed. The cells of filamentous

fungi *Aspergillus niger* entrapped into PVA cryogel appeared to be effective catalyst in their application for TCP biodegradation.

To obtain active immobilized biocatalyst, the fungus spores were initially immobilized into polymer structure. Then the granules were exposed in nutritional medium to germinate the spores and form active mycelium filling the inner free volume of porous PVA cryogel matrix. The process of formation of active biocatalyst was followed by control of mechanical characteristics of granules (Table 3). It was shown that filamentous fungus cells increased the solidity of biocatalyst granules and reinforced the polymer matrix.

The investigation of surface of biocatalyst granules with well-developed immobilized mycelium by SEM (Figure 6) confirmed that polymer matrix was completely penetrated by fungus cells. The prepared biocatalyst was successfully applied for degradation of TCP, obtained as a product of enzymatic hydrolysis of chlorpyrifos, and it was established that it can destruct 0.1 mM TCP per 24 h. The possible multiple use of the immobilized biocatalyst in the process of TCP degradation under batch conditions also was revealed in experiments.

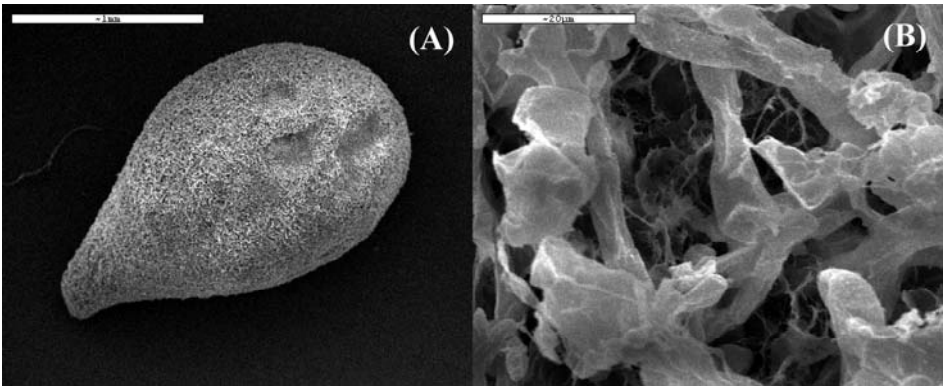


Figure 6. SEM of surface of biocatalyst granules with immobilized filamentous *A. niger* cells.

4. Biocatalytic Gel-Based Systems for Self-Defense

Enzyme His_6-OPH[25] was introduced into the gel system that was applied as a part of self-decontaminating defense material, containing three main layers, providing isolation of neurotoxic agents, detoxification the toxic compounds in case of their penetration into the inner parts of material and playing a role of hygienic barrier. Such complex material patented this year in Russia[26] allowed to considerably increase the defense degree against the CWA action and to get

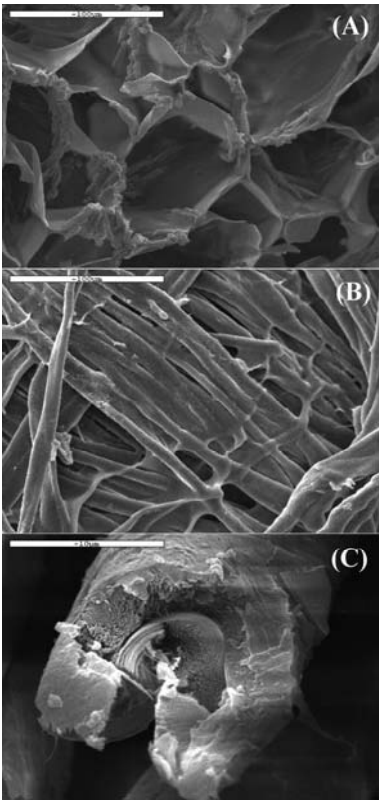


Figure 7. SEM of section of 7% sulphate chitosane gel highly cross-linked by glutaric aldehyde (A), coarse calico surface modified by sulphate chitosane gel composition (B) and section of cellulose fibers covered with protein-polymer composition (C).

as minimum 12-fold-prolongation of the material working time as compared to samples currently used in practice. It appeared that the catalytic system can be further activated by introduction of amines[27] into the sample of immobilized enzyme.

Immobilized biocatalyst based on OPH and porous fabric materials impregnated with chemically cross-linked sulphate gels was developed (Figure 7).

The fabric matrix possessed high sorption and water-retaining capacity, storage stability for at least 2 months and could effectively catalyze the hydrolysis of a wide spectrum of OPC. The immobilized enzymatic biocatalyst appeared to be very suitable form in the removal of OPC pollutions from various surfaces including skin.

Acknowledgements

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