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Review Article

Polymer Based Formulation and Immobilization Approaches in Enzymatic Detoxification of Organophosphorus Compounds: A review

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Abstract

Organophosphorus compounds are highly toxic chemicals with application in production of warfare agents and agricultural pesticides. Detoxification of areas that have been exposed to these types of materials is of vital importance and several approaches have been used for this purpose. Biodegradation of organophosphates is one of these approaches that is in the center of attention due to its high performance and environmental friendly nature. The key components in this approach are enzymes. Like many other industrial processes that employ enzymes, biodegradation of organophosphates has its own limitations. During recent years, researches have focused on addressing these limitations and devising new methods to overcome them. One possible method isimproving enzymatic formulations to gain decontamination systems with better detoxification efficiency. Immobilization has also been exercised in many ways to answer some of shortcomings in biodegradation of these compounds. Here, we will review enzymatic formulations of Organophosphates degrading enzymes with specific focus on polymer based approaches and immobilization techniques.

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Introduction

Organophosphates (OPs) include a wide group of chemicals that have different domestic and industrial uses. Examples of this group of substances include nerve gases (suman, sarin, tabun, VX), pesticides (malathion, parathion, diazinon, fenition, dichlorvos, chlorpyrifos and Ethion), and a number of other toxic chemicals [1].

OPs were first synthesized in the eighteenth century by Lassaigne. In 1854 Philip De Clermount explained synthesis of Tetraethyl Pyrophosphate in Congress of French Academy of Sciences. Eighty years later, two German scientists named Lange and Schrader began investigating the use of organophosphate as pesticides, but due to the high toxicity of these products (these days known as sarin and tabun) using them as pesticide was ceased.Instead, the German Defense Ministry put some changes on these products and used them as chemical weapons later in the World War I [2].

Since their development, attentions have been drawn to methods that can safely detoxify these compounds. Several mechanisms and various materials (ranging from physical and chemical based methods to biological approaches) have been used for this goal [3]. Generally, biological methods have attracted much attention for environmental friendly decontamination purposes. This "biodegradation" has been employed in many different circumstances and decontamination of different materials, from oil sludge to OPs [4, 5].

Enzymes are considered as the key components of biodegradation methods, however using them for this purpose, especially in industrial scale, is associated with different difficulties. One way to overcome obstacles in using enzymes and (at least in some cases) improve their activity is immobilization. The aim of this review is to discuss recent developments in the field of enzymatic immobilization in decontamination of OPs.

Organophosphorus compounds and their role as chemical warfare agents

Organophosphates (OPs) are considered to be among the deadliest chemical warfare agents (CWAs). OPs cause prolonged inhibition of cholinesterase (ChE). Following OP intoxication, inhibition of acetylcholinesterase (AChE), which is responsible for the degradation of the neurotransmitter acetyl-choline, leads to over-excitation of the cholinergic post synaptic receptors. This inhibition leads to a potential fatal cholinergic crisis that warrantsa nearly antidotal treatment. Chronic exposure to low dosage of OP might result a pathological sequel with

neuromuscular diseases. Butyrylcholinesterase (BChE) is another enzyme inhibited by OPs and is considered a potential target for reactivation (e.g.,using oximes) [6, 7]. One of the difficulties in dealing with OPs as a weapon is their environmental dispersal [5].

OPs that are used as pesticides, on the other hand,have fewer toxic effects than those of chemical weapons, but using them in high doses and in large quantities can cause similar effects. Statistics provided by the World Health Organization shows that about three million cases of poisoning with organophosphates or its derivatives occur annually, of which two hundred and twenty thousand cases have been fatal [8, 9].

These findings along with increase in using OP containing substances for different purposes brought a big challenge for environment and persons who are in contact with these compounds. Therefore, providing a safe and effective way for disposing OPs and decontaminating environment, individuals and equipment areof great importance.

Biodegradation of OPs

In the past few years, biodegradation has emerged as an effective and environmental friendly approach for decontaminating various sources of pollution from oil industry products and byproducts to chemical warfare agents and pesticides in industrial scale (Table 1).

Table 1. Advantages and disadvantages of biodegradation and chemical detoxification of OP compounds.

Method	Advantages	Disadvantages
Biodegradation	Environment friendly Non hazardous Lower logistic burden for large scale decontamination	Costly Sensitive to environmental changes Low substrate range
Chemical decontamination	Cheap High decontamination rate Effective on a wide range of toxins	Extremely toxin Corrosive

In this regard, enzymes such as Organophosphorus Hydrolase (OPH) and Organophosphorous acid anhydrolases (OPAA) originated from bacterial, fungal, and plant sources has been widely used in the detoxification of contaminated environments. So far, the use of these enzymes is done in three main ways; direct use of theenzyme (which usually embodies genetic manipulations to enhance the activity and specificity), using 'enzyme producing cells' that although solve the problem of lack of real-time enzyme production, but due to their low efficiency have little use in industrial level, and finally use of a cell that expresses the enzyme on its external surface. Each of these methods has its own advantages and shortcomings but in many circumstances direct use of enzymes is the method of choice because of versatilities in its application and ease of use [5].

As an example, Latifi *et al*., have reported the first application of thioredoxin (TRX) as an increasing agent for the expression level, solubility and stability of recombinant OPH enzyme. In particular, a significant correlation was observed between OPH expression, solubility, stability, and TRX [10].

In another study, comparison between periplasmic and cytoplasmic expression of OPH in *E. coli* has suggested that cytoplasmic expression system is much more effectivefor production of high amount of functional and accessible OPH in spite of inclusion body formation, which needs an additional refolding step [11].

Enzymatic degradation of OPs

Various enzymatic mechanisms have been studied for biodegradation of OPs. Based on the chemical reaction, this mechanism can be divided in two groups;hydrolysis and oxidation [5]. So far Most of the attention has been toward hydrolyzing enzymes and consequently there is a lot of information about them. In general, products of hydrolyzing reactions are roughly two fold less toxic than the raw material. These products are also much more sensitive to biological and chemical degradation methods than the original OP [12, 13]. One of the main concerns about choosing hydrolyzing enzymes is their substrate range. Most of hydrolyzing enzymes have a narrow substrate range which is therefore of great importance to be considered before are used. Among all of enzymes which are capable of hydrolyzing OPs, OPH and OPAA have the broadest substrate range. Moreover, due to stereo selective nature of enzymatic reactions, care should be taken for choosing hydrolyzing enzymes because some of routine OP degrading enzymes such as OPH (EC 8.1.3.1) and OPAA (EC 3.1.8.2) have a tendency toward less toxic stereoisomers of OPs [14].

Among bacterial enzymes, OPH from *P. diminuta* has the widest range of substrate specificity. OPH is composed of a dimer of two identical subunits containing 336 amino acid residues that folds into a (ab) 8-barrel motif [15, 16]. Many microorganisms can degrade OPs by hydrolyzing the compounds using OPAA,a family of single-polypeptide enzymes [12]. OPAA possesses low catalytic activity against P–O but high activity against P–F bonds [17]. Despite its potential in degrading OPs, OPH is not suitable for breaking P-S bonds. Peroxidases however are capable of conducting P-S breakage in substances like amiton and VX. The oxidative pathway in comparison with hydrolysis leads to formation of nontoxic and more environmentally benign degradation products [18]. Purified phenol oxidase (Laccase, EC 1.10.3.2) is the best known type of OP degrading oxidases. Amitai *et al*., have shown that use of Laccase along with the mediator 2,2 – Pazinobis (ABTS), results in complete and rapid degradation of the nerve agents VX and Russian VX (RVX) [18]. In this studya molar ratio of 1:20 for OP/ABTS and 0.05 M phosphate at pH 7.4 provided the highest degradation rate of VX and RVX.

Generally, oxidative enzymes are more effective and have wider substrate range than most hydrolysis enzymes in biodegradation of OPs. Moreover, studies show that oxidative enzymes (specifically laccase) are capable of degrading both optical isomers of OP compounds with same efficiency. This feature is especially important in biodegradation approaches because as previously mentioned, some of hydrolyzing enzymes have a tendency toward one type of enantiomers. The bacterial enzyme phosphotriesterase (PTE), for instance, exhibits stereo

selectivity toward hydrolysis of chiral substrates with a preference for the Sp enantiomer (naturally each chemical compound has two stereoisomers one of which is more toxic) [19]. Since the toxicity of different stereoisomers of OP compounds differs from each other, this feature of Laccase is very important for biodegradation purposes.

However, lack of empirical data on the oxidative enzymes on one hand, and chemical and catalytic sensitivities on the other hand has prevented the widespread use of this class of enzymes in bioremediation of OPs. Moreover, the utilization of fungal cells as a cell decontaminant may not be utilized for individual skin decontamination, since living cells cannot be utilized under the medical law [13].

Problems associated withformulation and immobilization of enzymes

Despite variety of applications in industry, using enzymes has its own challenges. Environmental sensitivity, specificity for certain types of substrates, production and purification limitations and high cost of using enzymes are some of these challenges.

Immobilization can greatly affect the stability of an enzyme. If the immobilization process introduces any strain into the enzyme, this is likely to encourage the inactivation of the enzymes under denaturing conditions (e.g. higher temperatures or extremes of pH). However, wherever there is an unstrained multipoint binding between the enzyme and the support, substantial stabilization may occur. This conformational problem is one the main concerns in immobilization or co-polymerization of OP degrading enzymes [20]. Another problem is so called "diffusional" effect. Preventing the enzyme molecules from interacting with each other is a necessary step for improving stabilization process. This effect is due to a combination of diffusional difficulties and the camouflage to enzymatic attack produced by the structural alterations [20]. Proper orientation of the enzyme, leakage of the enzyme during the decontamination process and non-specific binding are other important problems of using enzymes for biodegradation purposes. Researchers have overcome some of these difficulties by altering substrates (substrate engineering), modifying reaction system (medium engineering), or by enzyme engineering [21].

Polymer based enzymatic formulation

Current methods to stabilize OP degrading enzymes depend on immobilization, encapsulation, or mixing with hydrogel, fire-fighting foams, and polyelectrolytes.

However, the main problem in the field of enzymatic degradation of OPs which is developing a robust protocol that can preserve the enzyme activity and conformation and stabilize the enzyme under different working environments remains unsolved. One of the most recent advancements in this field is conjugation of enzyme with a polymeric block. Studies suggest that this Co-polymers provide advantages in comparison with native enzyme [22]. These improvements include enhancement in pH range, storage time, enzyme efficiency and higher thermos-stability. Co-addition of various types of polymers has been used forenhancing the stability of enzyme solutions. Among these, amphiphilic polymers are useful especially as surfactant formulations, satisfying the purpose of OP biodegradation [23].

In enzymatic formulations, surfactants play a vital role. Pluronic is the name of a family of surfactants that are commercially available witha wide range of molecular weights and block compositions, having the ability to spontaneously dissolve and self-assemble inwater. Due to this versatility in their properties, Pluronic offers a powerful potential for conjugation toenzymes [24].

Three-block copolymers (Pluronics) are biocompatible molecules composed of hydrophobic and hydrophilic blocks with different lengths. They have received much attention recently because of their efficiencyin targeted delivery of hydrophobic compounds. The unique molecular structure of pluronics facilitates the formation of dynamic micelles that are able to transport lipid soluble compounds. This feature has been exercised in using pluronic F-127 (a hydrophilic non-ionic surfactant of this family) to produce polymer based enzymatic formulation for bioremediation of OP compounds [23, 25].

In a study by Nagarajan *et al*., it has been demonstrated that amphiphilic poly[ethylene oxide-b-propylene oxide-b-ethylene oxide](PEO–PPO–PEO) three-block copolymers, known as Pluronics, can physically attach to OPH, and lead to improvements in both the stability and activity of the enzyme due to interactions between the hydrophobic block of Pluronic and hydrophobic amino acids in OPH. This approach of simply blending the enzyme with inexpensive, non-toxic, biocompatible, and commercially-available Pluronic provides an efficient formulation for OP detoxification with long pot life. Although surfactant molecules typically reduce the activityof enzymes, the addition of Pluronic F127 to OPH resulted in anactivity increase under many practically relevant conditions [23].

In another study by Suthiwangcharoen and Nagarajan, a facile approach to stabilize OPH using covalent conjugation with the amphiphilic block copolymer, Pluronic F127, leading to the formation of F127-OPH conjugate micelles, with the OPH on the micelle corona was reported (Fig. 1). Results of this interesting study shows a great promise for using Pluronic F-127 (and probably other surfactants with same polymeric properties or even blocks of Polyethylene Glycol (PEG)) as an carrier-immobilizer for increasing performance and stability of OPH [26].

The OPH in conjugate micelles exhibited reasonable improvement in activity and significantly enhanced stability, with elevatedheat, multiple freeze−thaw cycles, and different substrate conditions. Authors believe that the F-127 conjugation and the formation of micelles may provide spatial confinement to the OPH and promote a favorable OPH conformation, thereby enhancing the OPH stability.

To the best of our knowledge there are only two reports of using three-block pluronic based structures to stabilize OP degrading enzymes. Despite few other reports, results of these studies provide a great promise for using three-block polymers in OP biodegradation, even at industrial scale.

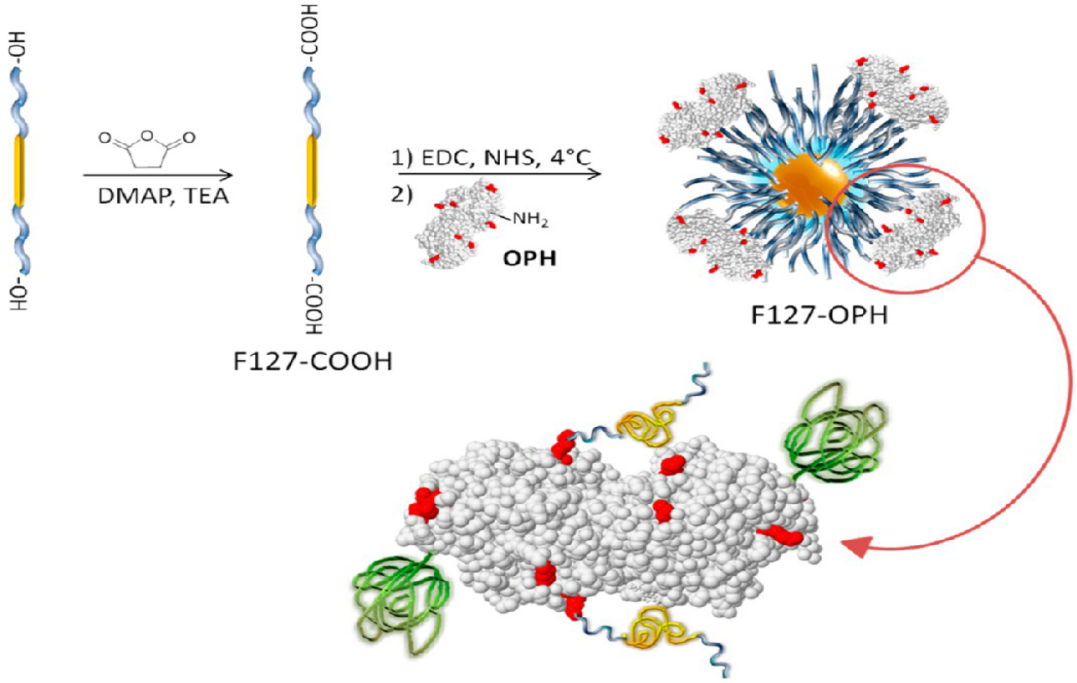

Figure 1. F127-OPH Conjugate Micelle [26].

Immobilization, an effective way to improve efficiency ofenzymatic OP biodegradation

Immobilization is a technical process in which enzymes are fixed to or within solid supports, creating a heterogeneous immobilized enzyme system. Immobilized form of enzymes mimics their natural mode in living cells, where most of them are attached to cellular cytoskeleton, membrane, and organelle structures. The solid support systems generally stabilize the structure of the enzymes and, as a consequence, maintain their activities. Thus, as compared to free enzymes in solution, immobilized enzymes are more robust and more resistant to environmental changes. In addition, heterogeneous immobilized enzyme systems allow the easy recovery of both enzymes and products, multiple reuses of enzymes, continuous operation of enzymatic processes, rapid termination of reactions, and greater variety of bioreactor designs. On the other hand, compared with free enzymes, most commonly immobilized enzymes show lower activity and, generally, higher apparent Michaelis constants because of a relative difficulty in accessing the substrate [27, 28].

In recent years, interest and high attention has been directed toward exploring the potential of immobilized enzymes [29]. Compared to their free forms, immobilized enzymes are generally more stable and easier to handle. In addition, the reaction products are not contaminated with the enzyme (especially useful in the food and pharmaceutical industries), and in the case of proteases, the rate of the autolysis process can be dramatically reduced upon immobilization [28, 30].

These alterations result from structural changes introduced into the enzyme molecule by the applied immobilization procedure and from the creation of a microenvironment in which the enzyme works, different from the bulk solution. The result would be a pure product not contaminated with other environmental ingredients and easy to be isolated from the solution. The attached enzyme is then ready for the subsequent reactions without the need for repeated, timeconsuming, and costly extraction and purification procedures [28].

In addition to biodegradation, immobilization has various applications in development of biosensors [21], especially in construction of biosensors for detection of contaminated areas. Glucose biosensors have been developed using electro spun PVA and surface-modified carbon nanotubes [22]. There are also various reports of using immobilized enzyme for detection of OP contamination. Construction of ampere-metric biosensor based on acetylcholinesterase (AChE) immobilized on CdS-decorated graphene (CdS-G) nanocomposite and many others based on OPH, OPAA or other enzymes are examples of this application of immobilization [23, 24].

Depending on the type of applied matrix, there are five main types of enzyme stabilization methods; Adsorption, Covalent bonding, Entrapment, Co-polymerization and Encapsulation.

Adsorption

Adsorption is the oldest and simplest method of immobilization of enzymes. In 1916, Griffin and Melson used adsorption technique for the first time to immobilize Invertase on the coal [31]. As the name suggests, in adsorption the enzyme is immobilized on the surface of the matrix mostly with use of weak physical bonds like hydrogenic, ionic or Van der Waals bonds. The carrier matrix can be made of inorganic, organic or synthetic materials.

Adsorption has been used for immobilization of bacterial cells that express OPH on their surfaces on the cotton matrices [32]. Results of this study showed that even after

death of bacterial cells decontamination process remains ongoing and the bacteria-cotton conjugate losses just less than 10% of its activity after 45 days.

Covalent bonding

In this method, immobilization is achieved by creation of covalent bond between thechemical groups of the surface of the enzyme and reactive groups of the support or carrier. In most of cases, this reaction needs previous activation of reactive groups of the matrix. Covalent bonding is one of the most used methods of immobilization and stabilization of enzymes. In addition to stabilization, this method has been used for purification purposes. A good example is attachment of cellulose-binding domain (CBD) to OPH and subsequent purification of this enzyme in a single step process using a cellulose containing matrix [33].

Mansee *et al*., used CBD-OPH to increase efficiency of OP degradation [34]. This study shows that when CBD-OPH is packed in acolumn bioreactor, it is able to completely degrade coumaphos up to a concentration of 0.2 mM. However, stirring of OPH immobilization cellulose materials resulted in complete OP degradation of 1.5 mM coumaphos. The bioreactor column degraded the compounds tested at high concentration, rapidly, and without loss of process productivity for about 2 months.

Gao *et al*., used highly porous nonwoven polyester fabrics to covalently immobilize organophosphate degrading enzyme A (OpdA) for organophosphate degradation. The fabrics were first activated with ethylenediamine to introduce free amine groups, andthe enzyme was then attached using the bifunctional cross-linker glutaraldehyde. The immobilization only slightly increased the Km (for methyl parathion, MP), broadened the pH profile such that the enzyme had significant activity at acidic pH, and enhanced the stability of the enzyme [35].

In another study covalent attachment of histidine6-tagged organophosphate hydrolase (His6–OPH) on Mesoporoustitania thin films resulted in good activity, and enhanced stability with respect to the free enzyme at extreme conditions of pH and temperature, especially around neutral pH and room temperature. His6–OPH was immobilized on mesoporous thin films with uniform (9 nm) and bimodal (13–38 nm) pore size distribution, through covalentattachment and physical adsorption [36].

Covalent immobilization has also been used for attachment of enzyme on bacterial spores. In one study, *Bacillus subtilis* spores were used as a new matrix for immobilizing OPH. Results of this study show that, in comparison free enzyme, both thermal stability and activity of immobilized OPH is increased in comparison free enzyme [37].

Entrapment and Encapsulation

In this method the enzyme is physically trapped within the supporting matrix. Type of the link between the enzyme and support can be covalent or non-covalent. Pore size and membrane permeability of support matrices highly depends on the type of material of support and is completely adjustable in many cases. Various types of materials are available to beused in this method, such as cellulose, polyacrylamide gel, agar, gelatin, etc. This method has also been usedin the case of OP-degrading enzymes. For example, Gill *et al*., have investigated *P. dimunita* organophosphate hydrolase immobilized in sol-gel polymers and enzyme-polymer composite materials. They developed a sol-gel encapsulation technique that employs poly[glyceryl silicate] (PGS) rather than the conventional poly[methyl silicate] (PMS). When theycompared the efficiencies of OPH immobilized in sol-gel materials with OPH immobilized in polyurethane foam, they observed high activity retention in the PGS-derived sol-gel (94%) and polyurethane foam (68%), whereas the sol-gel prepared using PMS had activity retention of only 28%. All three preparations had good stability over 700 h at 40°C, with the PGS sol-gel performing best after long time periods [38, 39].

In another study, Lu *et al*., reported the synthesis of OPH nanocapsules that are highly active and robust using a simple two-step process. In this way the polymershells can effectively stabilize the interior OPHs while enabling rapid substrate transportation, affording a novel class of biocatalytic nanocapsules with outstanding activity and stability for various applications. These applications include using nanocapsule in aqueous solution, blending it with foams and its fabrication on various polymer structures including cellulose to form biocomposites [40].

Co-polymerization

Like covalent bonding, co-polymerization has been widely used and studied for OP biodegradation purposes. In this method, which is also known as cross-linking, immobilization happens with direct linkage of the enzyme to the multifunctional groups of the matrix. These groups are usually glutaraldehyde or diazonium salts.

Generally, any enzyme that is present in theaqueous solution can participate in the polymer synthesis via the lysine residueson the surface, effectively creating an enzyme-containing polymer network withmulti-point attachment [41, 42]. Havens & Rase were the first to investigate the incorporation of OPH into a polyurethane sponge [43]; this approach has also been studied extensively in our laboratory [43, 44]. A detailed kinetic analysis of OPH incorporated into polyurethane foams showed that no internal or external diffusion limitations exist in aqueous media. Furthermore, up to 50% of activity retention was observed with a modest increase inthe KM from 0.047 mM to 0.124 mM [43]. The multipoint covalent attachment ofthe enzyme-polyurethane affords very high stability, increasing enzyme half-lifefrom 1.8 days for soluble enzyme in buffer at ambient conditions to 278 daysfor the immobilized preparation [45]. The enzyme-containing polyurethane also had increased thermos-stability at 50°C, increased resistance to proteolytic attack, and increased resistance to buffered bleach solutions when compared to soluble enzyme [45, 46]. LeJeune *et al*., also prepared polyurethane foams containing AchE, reporting that 90% of available enzyme activity was retained within thepolymer during synthesis. The AchE-foams were highly active after storage fortwo full years [46, 47].

Conclusion

Using polymersas additives for enhancing properties of OP degrading enzymes has a short history. However, results of studies show that these methods have a great potential to

become popular in the field of bioremediation of Neurotoxins and pesticides that have been developed based on the OP formulations. Likewise, immobilization of enzymes are powerful means to improve their performance and stability. Recent developments in the field of material science and ongoing studies on methods and techniques of immobilization promise a bright future for using this approach in a wide range of applications especially in industrial biodegradation of OP compound like reservoirs of nerve agents, decontaminated areas, equipment and people and detoxification of insecticides and pesticides.

In addition to co-polymerization and immobilization, direct change of enzyme properties by using genetic engineering and medium engineering are two important areas of research in enzymatic biodegradation. These three approaches have provided a unique opportunity for a safe and effective way of decontamination of OP-base warfare and toxins. Prospect of further advance in this field provides a great promise for use of biodegradation in industrial scale for detoxification of one of the most widely used chemical toxins.

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