

## 78. OP NERVE AGENT DECONTAMINATION, DETOXIFICATION, AND DETECTION USING POLYURETHANE IMMOBILIZED ENZYMES

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### ABSTRACT

As an extension of the bioscavenger approach to the protection against organophosphate toxicity, we developed a sponge product, composed of polyurethane immobilized ChEs (AChE and BChE) and organophosphate hydrolases, and oxime for decontaminating OPs from sensitive biological surfaces. The ChE-sponge is also a biosensor for OPs so troops can rapidly determine OP exposure and contamination. The enzyme products exhibit remarkable mechanical and chemical stability when immobilized and do not leach from the synthesized matrix, yet retain the function of their soluble counterparts. For example, DFP and MEPQ reacted with the immobilized ChEs, and rinsing the sponge with HI-6 restores cholinesterase activity, permitting the AChE-sponge to be recycled many times. Since OPs need to be wiped onto the sponge to be detoxified, several sponge formulations have been developed to rapidly remove soman from guinea pig skin. Using this enzyme-sponge technology, we are developing a rapid and simple kit to detect OP contamination on humans, in water or almost any environment. ChEs and non-ChE enzymes have been immobilized to yield small OP sensitive and selective biosensors. For long-term OP detection, ChE-biosensors were continuously exposed to untreated natural fresh or salt water over 60 days at room temperature: the badges retained 80% of their original activity. In conclusion, immobilized ChEs retain high activity and increased stability, making them suitable for a variety of detoxification and decontamination schemes for both chemical weapons and pesticides directed against ChEs, and as biosensor badges to immediately detect or monitor long-term OP contamination, for example in drinking water.

### INTRODUCTION

It was previously demonstrated that a variety of enzymes exhibited enhanced mechanical and chemical stability when immobilized on a solid support, producing a biocatalyst. The study of degradation of organophosphates by immobilized enzymes dates back to Munnecke (1), who attempted to immobilize a pesticide detoxification extract from bacteria by absorption on glass beads. The absorbed extract retained activity for a full day. Wood and coworkers (2), using isocyanate-based polyurethane foams (Hypol®), found that a number of enzymes unrelated to OP hydrolysis could be covalently bound to this polymer; after that Havens and Rase (3) successfully immobilized parathion hydrolase. More recently, the enzyme bioscavenger approach (4, 5) has been shown to be effective against a variety of OP compounds *in vitro* and *in vivo*; pretreatment of rhesus monkeys with fetal bovine serum (FBS) acetylcholinesterase (AChE) or equine serum butyrylcholinesterase (BChE) protected them against a challenge of up to 5 LD<sub>50</sub> of soman. While the use of cholinesterase (ChE) as a single pretreatment drug for OP toxicity provided complete protection, a stoichiometric amount of enzyme was required to neutralize the OP *in vivo*. To increase the OP/enzyme stoichiometry, enzyme pretreatment was combined with oximes such as HI-6 so that the catalytic activity of OP-inhibited AChE is rapidly and continuously restored before irreversible aging of the enzyme-OP complex can occur. Thus, the OP is continuously detoxified. Based on the two above observations, (a) that polyurethane foams are excellent adsorption materials for OPs such as pesticide vapors (6), and (b) that soluble ChEs and oxime together have the ability to detoxify OP compounds, we combined these features in a porous polyurethane foam formed *in situ* from water-miscible hydrophilic urethane prepolymers and the enzymes.

Detection of OP compounds is of paramount importance to prevent casualties due to OP exposure and cholinergic crisis. Traditional analysis of cholinesterase inhibitors is performed using gas and liquid chromatography and mass spectrometry (7). More problematic is the measurement of nerve agents in mixtures, which require extraction procedures and manipulations. These instrument intensive techniques have significant drawbacks when considering an individual kit for field deployment, including lack of portability, simplicity, cost, reliability, and rapid results. An alternate technology is a biosensor. Biosensors (8) have been widely used to detect biological, pharmacological, or clinically important compounds. Enzyme sensors have the advantage of selectivity, sensitivity and, most important, specificity, ease and portability, and markedly simplified instrumentation. Enzyme sensors can behave as a dosimeter, accumulating only those inhibitors demonstrating exquisite selectivity for the specific enzyme, while ignoring all other environmental interference. A variety of biosensors based on cholinesterases

immobilized non-covalently have been described (9). The drawback to these methods includes lack of enzyme stability at ambient conditions once opened, leaching from the surface to which it was non-covalently deposited, sensitivity to denaturing conditions, and short half-life when in solution. The currently fielded spot detector, the M256A1 chemical agent detector kit, has a dry eel ChE non-covalently applied (dried) onto a fiber: it can only be exposed to and monitor air or vapor for OPs. The major advantage of the immobilized ChE-sponge is that the enzyme will not leach from the polyurethane support so that the OP ticket or badge can be used to sample anything from soil, water, to air.

We envision a reusable immobilized enzyme sponge of cholinesterases and oximes for OP decontamination. Also, the immobilized polyurethane enzymes will make versatile biosensors for detecting organophosphates in all environments.

## METHODS

**Sponge synthesis and assay:** The immobilized enzyme-sponge can be synthesized and cured in less than 20 minutes at ambient temperature, molded into the shape of any container. A new technique (10) was developed to mix the prepolymer (Hypol prepolymer TDI 3000, Hampshire Chemical, Lexington, MA) and enzyme in buffer containing 1% surfactant (Pluronic P-65, BASF Specialty Chemical, Parsippany, NJ). This method replaces the rapid mixing by an electric drill with a mixing stator (a stationary plastic disposable tube for two-component mixing) to effectively reduce high shear stress and partial denaturation of the enzymes during mixing of the two components, prepolymer and enzyme. In addition to simplicity and easy scale-up, the activity of ChEs coupled to the prepolymer increased about two-fold with the mixing stator compared to the high-speed drill mixing. The decontamination sponge containing the immobilized enzymes molded in a Tupperware® container the size of a human hand is shown in Figure 1 (left), while a sensor sponge, about 1 cm in diameter, is illustrated on the right. The sponges were evaluated for activity, such as temperature and environmental resistance, inhibition by OPs and reactivation by oximes, and stability using the appropriate technique for each enzyme, e.g., the modified Ellman method for ChEs.

## RESULTS

Previously, we demonstrated (10) that immobilized ChEs showed little leakage from the sponges even after many washes over several days, and therefore were irreversibly and covalently incorporated into the polyurethane matrix. The enzymes attach covalently at surface lysines to the inert foam at multiple points during the polymerization process, thereby becoming an integral part of and acquire the structural integrity of the resulting polymerized matrix. The resulting ChE-sponge, a matrix of TDI polyurethane and enzyme, results in remarkable stability of these immobilized enzymes; ChE-sponges retained their original activity after more than 3 years at 0°C, most of their activity after 1.5 years at 25°C, and more than 50% activity after 1.5 years at 45°C.

Our recent results demonstrate the following additional characteristics of sponges containing immobilized AChE. We evaluated different polymers for immobilization of the enzymes. Originally, we used tolyl diisocyanate (TDI, 5%) and methylenediphenyl diisocyanate (MDI, 5%) polyether prepolymers. The TDI prepolymer proved more suitable to enzyme immobilization, presumably due to its flexible structure, and the TDI yielded ChE-PUFs with enhanced resistance to environmental denaturation. We have now evaluated prepolymers containing 3% TDI and 5% isophorone diisocyanate. The latter prepolymers yielded about the same efficiency for covalently coupling AChE and retained a high degree of esterase activity. We also describe here that the OPs diisopropylfluorophosphate or MEPQ (7-(methylethoxyphosphinyloxy)-1-methylquinolinium iodide) inhibited the activity of ChE-sponges, as was observed for non-immobilized ChEs in solution. The oxime HI-6 restored activity of the OP-poisoned AChE-sponge until the molar concentration of MEPQ reached approximately 1000 times that of the cholinesterase active site. However, the AChE-sponge could be recycled many times by rinsing the sponge with HI-6 in the absence of OP. In this case, most of the original cholinesterase activity could then be restored to the sponge. We now find that the ability of the immobilized enzymes and HI-6 to detoxify the OP MEPQ was dependent upon the efficiency of the sponge to decontaminate particular surfaces. The sponge alone could remove/decontaminate MEPQ from non-porous plastic surfaces (>97%), and an AChE-sponge with HI-6 completely detoxified the removed MEPQ. However, the sponge without enzyme was not more effective than the M291 decontamination kit for removing neat soman applied to guinea pig skin. We therefore evaluated additives to the polyurethane matrix, both during synthesis and post synthesis, to improve the removal and extraction of OPs from guinea pig skin. These experiments were performed with sponges lacking enzyme so that we could directly evaluate the ability of the sponge to decontaminate the skin. The prepolymer was not altered since currently there is no formulation of Hypol prepolymer with an increased hydrophobic nature that might be expected to absorb the OP more effectively. Liquid additives possessing surfactant properties, zwitterion and buffers, and partial organic solubilizing characteristics were tested, including centrimide, 18-Crown-6, iso-octane, kryptofix 222, polyethylene glycol 6000, triacetin, and tetraglyme.

We found that most solutions provided no significant benefit over the original phosphate buffer included in the sponge that was optimized for enzyme activity. However, both triacetin and tetraglyme provided additional ability to remove soman from the skin, protecting guinea pigs about four to five-fold better than the M291 kit (Table 1). In addition, sponges were synthesized so that activated carbon would be incorporated into the polymer matrix. The addition of carbon did not interfere with the immobilization of ChEs. However, while carbon-sponges alone provided mixed results for removing soman from skin, they might be effective in removing other toxic agents. Sponges containing 2-PAM and HI-6 also showed increased protection to soman skin toxicity compared to the M291 kit, at least four-fold (Figure 2).

We have also prepared small polyurethane badges/sensors approximately 1 cm in diameter in which the cholinesterases (FBS AChE and/or equine BChE) are covalently immobilized, as shown in Figure 1. The badges composed of mammalian AChE or BChE have similar kinetic properties to soluble enzyme and selectivity to OPs. Most important, the OP MEPQ inhibited either forms of the enzymes, soluble or immobilized, at the same rate as determined by their bimolecular rate constants (Table 2).

We have developed alternate indicator systems for the ChE badges: OP-exposed badges can be evaluated qualitatively by visual color changes or luminescence for dark-adapted eyes. The latter reaction uses a coupled reaction of immobilized ChE, choline oxidase, and peroxidase. Like the ChEs, choline oxidase was successfully immobilized to the prepolymer using the same mixing apparatus. We found that soluble and immobilized choline oxidase activity had similar kinetic parameters, and the pH dependent activity of the soluble or immobilized choline oxidase was identical. Since the optimal pH for ChEs and choline oxidase were the same (about pH 8), the coupled reaction in the assay could be simultaneously optimized for both immobilized enzymes. In another indicator reaction, we used Amplex Red, a reaction that generates an intense red color that is also fluorescent. The fluorescent chromagen yielded about a 4000-fold increase in sensitivity compared to the visible red chromagen. This fluorescent indicator would be particularly useful in a hand-held fluorometer, which could provide quantitative values of ChE inhibition instead of qualitative information.

One significant difference and advantage the immobilized enzyme sensor has over the current M256A1/M272 chemical agent detector kit is that immobilized enzymes do not dissociate (leach) from the sensor. Therefore, the immobilized enzyme sensor can be used to test water or even left in the liquid source for extended periods. To develop this concept, we prepared sensors using purified AChE and BChE and exposed them continuously to different pHs between 4-10.5 for over 2 months at 25°C. The badges showed good retention of enzyme activity and could remain at pH 6-8 in aqueous solution for more than 60 days. However, at the pH extremes of 4 and 10.5, the badges were sensitive to pH-induced inactivation, since a recovery period at pH 8 restored <20% of the original activity of the badge. It is known that both cholinesterases in solution are rapidly and irreversibly inactivated below pH 5 and above pH 9.5. Thus, immobilization affords only partial protection. In another situation, we placed the ChE sensors in natural water samples at room temperature (Figure 3, fresh water from the Allegheny River, PA). The sensors retained high activity even if the samples were not autoclaved, indicating that the immobilized enzymes were resistant to microbial degradation. In contrast, the M272 kit designed for water samples retained activity for minutes, rather than more than a month for the immobilized sensor (Figure 3). Therefore, the immobilized enzyme badges/sensors could be placed in the natural environment such as dirt to detect OPs, and then rinsed with local water to remove any interfering material before initiation of the color reaction to determine if OPs are present.

## CONCLUSIONS

In conclusion, the capability to decontaminate personnel is extremely valuable to the military. The system must be lightweight, nontoxic to personnel, highly efficient, and compatible with sensitive medical areas such as eyes. In addition to decontamination of skin and personnel, the enzyme-sponges can be utilized for preventing cross-contamination of medical and clinical personnel. Still more uses for these formulations could include decontamination foams as masks and in garments, augmenting carbon filters that absorb OPs without inactivating them. OPs in the environment could be contained and decontaminated if the ChE-sponges were incorporated into fire-fighting foams. The enzyme-sponges could be used to decontaminate sensitive equipment without posing new environmental disposal problems, since the final products are rendered inert. Indeed, the sponges should be suitable for a variety of detoxification and decontamination schemes for both chemical weapons and civilians exposed to pesticides or highly toxic OPs such as sarin. This is important since currently accepted methods for decontamination of personnel, large areas, and materials use sodium hydroxide and bleach, which are caustic and harmful and also pose a significant environmental burden. In addition, the ChE-sponges could be used in chemical-biological sensors and incorporated into electronic hand-held telemedicine devices, for instance as electrochemical OP probes. The immobilized enzyme sensors have the unique ability, unlike the current OP ticket, to detect OPs in any environmental condition, such as vapor, water, or even soil, and in circumstances requiring long-term remote

sensing. Also, the immobilized sensors could be developed into a differential OP detector to identify the type of OP contamination, which would aid in treatment. With the constant threat of chemical warfare, terrorist acts, or spill of pesticides, the development of alternative means of protecting and decontaminating individuals from exposure to OP nerve agents is critical.

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#### KEYWORDS

Decontamination, Detoxification, Immobilized enzymes, Cholinesterases, Organophosphates, Biosensors

#### FIGURES AND TABLES

**Table 1.** Sponge Additives Protect Soman Contaminated Guinea pigs

<b>Additive to sponge</b>	<b>LD<sub>50</sub></b>	<b>Protective Ratio</b>
HI-6 (oxime)	79	8.0
2-PAM (oxime)	76	7.7
Tetraglyme	88	8.9
<b>Reference Values</b>		
M291 decontamination kit	17.7	1.8
Soman alone	9.9	

**Table 2.** Effect of Immobilization on ChE Kinetic Parameters: Time-Dependent Inhibition of ChEs by the OP MEPQ

<b>ChE</b>	<b>Enzyme Form</b>	<b>Bimolecular rate constant (M<sup>-1</sup> min<sup>-1</sup>) ± SD</b>
FBS-AChE	soluble	1.59 ± 0.52 x 10 <sup>8</sup>
	immobilized	1.00 ± 0.28 x 10 <sup>8</sup>
Equine-BChE	soluble	4.15 ± 0.78 x 10 <sup>7</sup>
	immobilized	4.21 ± 2.00 x 10 <sup>7</sup>