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Monitoring of Diisopropyl Fluorophosphate Hydrolysis by Fluoride-Selective Polymeric Films Using Absorbance Spectroscopy

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Abstract

In this study, a novel system for the detection and quantification of organofluorophosphonates (OFP) has been developed by using an optical sensing polymeric membrane to detect the fluoride ions produced upon OFP hydrolysis. Diisopropyl fluorophosphate (DFP), a structural analogue of Type G Chemical Warfare Agents such as Sarin (GB) and Soman (GD), is used as the surrogate target analyte. An optical sensing fluoride-ion-selective polymeric film was formulated from plasticized PVC containing aluminum(III) octaethylporphyrin and ETH 7075 chromoionophore (Al[OEP]-ETH 7075). Selected formulations were used to detect the fluoride ions produced by the catalytic hydrolysis of DFP by the enzyme organophosphate hydrolase (OPH, EC 3.1.8.1). The changes in absorbance that corresponded to the deprotonated state of chromoionophore within the film results from simultaneous co-extraction of fluoride and protons as DFP hydrolysis takes place in the solution phase in contact with the film. The developed sensing system demonstrates excellent sensitivity for concentrations as low as 0.1 μ M DFP.

Introduction

Organofluorophosphonates (OFPs) belong to a subclass of organophosphates (OPs) with anti-cholinesterase properties [1]. The presence of fluorine as the leaving group differentiates OFPs from other OPs with O, S, or CN as the leaving groups following chemical hydrolysis. Among the most notorious chemical warfare agents, Sarin and Soman both belong to the OFP subclass, known as GB and GD agents, respectively. Consequently, significant effort is being directed into the research and development of relatively simple sensors for their detection in the environment. Some of the conventional OFP detection systems include chromatographic techniques interfaced with mass spectrometers [2], surface acoustic wave sensors based on changes in properties of a piezoelectric crystal upon the

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adsorption of specific analyte [3], and molecular imprinting techniques in combination with spectrometers using a europium probe to measure the hydrolysis products of Sarin et al. [4]. As such, these techniques offer good sensitivity; however, most of these sophisticated technologies have limited portability, and their complex operation requires skilled personnel. In lieu of these approaches, the employment of enzyme-based biosensing systems may be a less expensive alternative with better operational abilities for field detection purposes. In previous studies, the inhibition of acetyl-cholinesterase was used as a means to detect and quantify neurotoxic OPs providing good sensitivity [5]. However, their limits in application included poor selectivity and limited reusability attributed to the nearly irreversible AChE enzyme inhibition. A more specific biorecognition element such as organophosphorus hydrolase (OPH, E.C. 3.1.8.1) that catalyses the hydrolysis of various OPs with dramatically different kinetic parameters has the potential to discriminate certain OPs against other cholinesterase inhibitors [6–8]. For example, the K_{cat} values at 7.0 pH and 25 °C of OPH from *Pseudomonas diminuta* that hydrolyzes a variety of OPs such as paraoxon (P-O), DFP (P-F), Soman (P-F) and Sarin (P-F) was found to be 2070 s⁻¹, 41 s⁻¹, 4.8 s⁻¹ and 56 s⁻¹ respectively [9]. Extensive studies using site-directed mutagenesis have shown tailored catalytic activities towards substrates having different bonds [10]. There is a related enzyme, organophosphorus acid anhydrolase (OPAA, EC 3.1.8.2) that is specific towards OFP (type G) hydrolysis [11] which has the potential to selectively detect OPFs over other OPs.

The biosensors based on OPH or OPAA reported to date, which are specific for OFPs, chiefly rely on the generic detection of protons [12–14] and/or the specific detection of fluoride ions [15] generated upon hydrolysis of the target species. Proton detection as a means for OFP quantification suffers from several disadvantages. First, pH-based detection systems are largely influenced by the buffering capacity of the target analyte sample. High buffering capacity of the sample may prevent the ability to monitor changes in proton activity, and poorly buffered samples may render the system unstable. Hence, extremely good referencing techniques are crucial in order to avoid false positive signals, and they are difficult to sustain. Secondly, all OPs, regardless of their leaving groups or their application as pesticides or chemical warfare agents (CWAs), produce hydrogen ions upon hydrolysis, thereby creating challenging selectivity issues. Addressing the selectivity requirement of an OP biosensor is critical to differentiate the presence of a pesticide such as parathion or acephatae from a chemical nerve agent such as Sarin or Soman. This discrimination is critical in order for the local emergency planning committees (LEPC) to act appropriately for remediation and prevent public panic. Thirdly, the reuse of enzyme-based sensors over extended periods is limited by the loss of their enzymatic activity requiring constant recalibration (and even during) detection analysis. These issues may be addressed by developing a system that combines the advantages of immobilized enzymatic hydrolysis and selective fluoride ion sensing devices. One such development involves the use of a commercially available pF ion-selective electrode coupled to OPH immobilized onto a silica gel in a batch mode set-up [12]. The system however suffers from poor sensitivity.

Recent studies of fluoride detection systems based on polymeric films have demonstrated that thin films prepared with Al(III) porphyrins in conjunction with a lipophilic pH indicator such as dibromofluorescein can be employed to create a highly sensitive and reusable fluoride ion sensor based on the coextraction principle [16]. In this preliminary study, we show that it is possible to develop a stable, enzyme-affiliated OFP sensing system by utilizing such a fluoride ion-selective optical film to transduce the OFP hydrolysis by OPH into a measureable signal. The interfacing of an Al[OEP]-ETH 7075 ion-selective membrane with an enzyme-based measurement greatly widens the window of dynamic range of detection. The principle of OFP detection in the current study is shown in Scheme 1.

Diisopropyl fluorophosphate (DFP), is a structural analogue of the class-G chemical warfare agents and is used as the model target analyte in these studies. Here, DFP undergoes catalytic hydrolysis in the presence of OPH to give equimolar concentration of fluoride ions. The generated products are exposed to fluoride sensing polymer film coated on quartz slide resulting in the coextraction of fluoride ions and protons from solution phase by the aluminum porphyrin and chromoionophore (ETH 7075), respectively, into the film. This coextraction results in the protonation of ETH 7075 which is measured as a change (decrease) in absorbance at λ_{max} of the deprotonated form of the chromoionophore. The entire set-up of the sensing film with analyte solution was contained in a standard 1 cm cuvette (see Figure 1).

Experimental

Materials

Octaethyl porphyrin (OEP) was purchased from Frontier Scientific (Logan, UT) and aluminum chloride (AlCl_3) was purchased from Strem Chemicals, Inc. (Newburyport, MA). Diisopropyl fluorophosphates (DFP), 4',5'-dibromofluorescein octadecyl ester (ETH 7075), 2-nitrophenyloctylether (o-NPOE), poly(vinyl chloride), sodium fluoride, benzonitrile and tetrahydrofuran was purchased from Sigma Aldrich (St. Louis, MO). L-Glycine, phosphoric acid and quartz slides were purchased from Fisher Scientific (Pittsburgh, PA). Ches (N-cyclohexyl-2-aminoethanesulfonic acid) was purchased from Alfa Aesar (Ward Hill, MA). Wild-type OPH (E.C. 3.1.8.1) from recombinant *Escherichia coli* was purified at Texas A&M as described elsewhere [17]. De-Ionized water (18.2 M Ω cm) was used from Millipore water purification system for the preparation of buffer solutions.

Instrumentation

UV/vis spectrophotometer (Ultraspec™ 2100 pro, Amersham Biosciences, Piscataway, NJ) was used for all absorbance measurements. The schematic of the experimental set-up comprising of a cuvette with sensing film is shown in Figure 1. The slide coated with the polymer film is placed in a standard 1 cm cuvette containing the analyte solution (Figure 1). A slot with inside grooves made on the cuvette cap was used to hold the slide in a position such that it was perpendicular to the light path. The arrangement was sturdy enough to prevent the movement of slide during handling and experimentation. The whole system was in turn placed in the standard cuvette holder of the spectrophotometer for measurement purposes.

Preparation of Aluminum octaethyl porphyrin (Al[OEP])

The Al[OEP] was synthesized by refluxing 0.5 mmol OEP and 2 mmol aluminum chloride in 25 ml benzonitrile overnight under nitrogen atmosphere. Following the evaporation of benzonitrile, the crude product was dissolved in methanol and precipitated in 3 M hydrochloric acid solution. The collected precipitate was further washed with 3 M hydrochloric acid. Purification of the crude product was achieved by flash chromatography on silica using dichloromethane as the solvent. The final product was characterized by mass spectrometry yielding a desired molecular weight peak at $m/z = 591.3$ and using UV/Visible spectrometry.

Preparation of ALOEP-ETH 7075 cocktail

The ALOEP-ETH 7075 optical fluoride sensing film was prepared using the procedures reported by Badr and Meyerhoff [18]. The most effective formulation as discussed by Badr et. al. resulted from the mixture of 2.14 mg of Al[OEP], 2.08 mg ETH-7075, 30 mg of 2 nitrophenyloctylether, and 15 mg of poly(vinyl chloride) with 1 ml of tetrahydrofuran [18].

Complete dissolution of the reagents and mixing was ensured for Al[OEP]-ETH 7075 using sonication. The cocktail was then sealed in a glass vial and stored at 4°C until use. Cocktails were used within 3 days of their preparation.

Preparation of slide coated with polymerized film

Quartz slide was cleaned using acetone prior coating with polymeric films. “Doctor blade method” was used to coat 20 µl of Al[OEP]-ETH cocktail on each of the cleaned slides. The slides coated with the optical sensing film were allowed to dry, and then stored in dark until use.

Procedure

For the detection of DFP, various concentrations of DFP were formulated in CHES buffer, pH 8.5, and were completely hydrolyzed by treatment with 10 µl of ~ 7 µg/ml of OPH for 2 min. The reaction solutions were then further diluted in glycine phosphate buffer (2.0 M, pH 3.0) such that the final concentrations of DFP in contact with Al[OEP]-ETH 7075 doped film were in the range of 0.1 µM, 1 µM, 10 µM, 25 µM, 50 µM, 75 µM and 100 µM. The absorbance spectra of the polymer films in contact with the hydrolyzed/diluted DFP solutions for 10 min were recorded on a UV-vis spectrophotometer (Ultaspec 2100pro, Amersham Biosciences). The absorbance peaks corresponding to the chromoionophore’s deprotonated state were monitored for changes and plotted against the various DFP concentrations. This two step procedure of hydrolysis and detection was similar to the studies performed for glucose sensing using the Al[OEP]-ETH 7075 polymer films [19]. To reference against possible non-enzymatic hydrolysis of DFP over the same assay time, Al[OEP]-ETH 7075 films were also exposed to samples that were not hydrolyzed by OPH.

The percentage changes in the absorbance (%A) were calculated as follows:

$$\%A = \left[\left(\frac{A_o - A_x}{A_o} \right)_{DFP-OPH} - \left(\frac{A_o - A_x}{A_o} \right)_{DFP} \right] \times 100 \quad [1]$$

where, A_o is the absorbance of the polymer film coated slide in glycine phosphate buffer solution and A_x is the absorbance of the slide when treated with “x” M DFP.

Results and Discussion

The use of Al[OEP]-ETH 7075 polymeric film on quartz slide to detect fluoride ions has been discussed in detail by Badr et. al [18]. Studies have shown its application for glucose sensing by coupling enzyme-based reactions with these polymeric films on microtitre plate wells [19]. Since OPH is rendered inactive due to the protonation of its active site [20] at pH 3.0, the hydrolysis and detection of DFP is carried out in two steps under different pH conditions similar to the procedure followed by Robboh et. al for glucose detection [19].

Although OPH activity has its highest substrate specificity with phosphotriesterase (P-O) bonds, its hydrolyzing capability towards OFPs is well established [12–15]. As shown in Scheme 1, complete hydrolysis of DFP results in the production of equimolar concentration of fluoride ions. Figure 2 shows the absorbance spectrum of the polymeric films treated with various concentrations of DFP. From the figure, the absorbance peak corresponding to the deprotonated form of the pH chromophore was found to be 538 nm. It should be noted that changes in the 380–400 nm range, correspond to spectral changes of the Al[OEP] species due to dimerization of the Al[OEP] species upon interaction with the extracted fluoride ions [18]. This spectral region can also be utilized for analytical purposes, although there is potential for more interferences in this region when examining real-world samples. A single

optical polymer film was used to test the various concentrations of DFP samples hydrolyzed by OPH for a single experimental data set. Another fluoride sensing film was used as a control for DFP samples with similar concentrations that were not hydrolyzed by OPH. The % change in absorbance (y) as calculated using equation 1 for figure 2 for various concentrations (x) can be represented by the following equation.

$$y=0.4086 x+6.465 (R^2=0.966, n=3) \quad [2]$$

The response was nearly linear in the range of DFP concentrations (100 nM – 100 μ M), over which the sensing system was tested (Figure 2). Concentrations as little as 0.1 μ M were readily detectable, based on the percentage change in signal for sample treated with OPH that was at least three times greater than the percentage change in signal obtained for polymer film that was exposed to DFP solution not treated with OPH. This detectable concentration was much lower than those obtained using earlier DFP biosensor studies with pH FETs (20–25 μ M DFP) [15] and optical waveguides (1–10 μ M DFP) [13, 14]. This level of DFP detection is below the oral lethal dosage limits in humans (~21 μ M) for DFP and dermal lethal dosage limits for GB (0.7 μ M) and GD (0.5 μ M) nerve agents. The sensor is however restricted by the slow response times governed by the fluoride detection step.

It should be noted that in the current configuration, the enzymatic reaction requires a high pH to be efficient (pH 8.5) while the sensing chemistry of the polymer film requires low pH (pH 3.0). Hence, optimal analytical capabilities are only achieved when the reaction and detection process is carried out in two separate steps. However, it may be possible to ultimately overcome this limitation by locally changing pH values near the sensing film using electrochemical methods to generate protons [21] or by encapsulating the enzyme in silica matrix with appropriate surfactants to protect it against extreme acid conditions [22].

Conclusions

The utility of enzyme-based, catalytic hydrolysis of organophosphosphate neurotoxins that releases fluoride ions in conjunction with an amplifying fluoride optical sensing film is demonstrated here as a potential approach to overcome the current shortcomings for the development of OFP-specific sensing systems. The combined sensing strategy (catalytic DFP hydrolysis monitoring by fluoride selective polymeric films) uses simple absorbance spectroscopy to enable the detection of sub-micromolar quantities of analyte, a significant improvement over earlier OFP detection systems. The introduction of chromoionophore for monitoring absorbance changes at ~ 538 nm for the presence of fluoride ions enables the potential use less expensive optical substrates such as commercially available glass slides and those coated with metal oxides as opposed to the expensive quartz slides, as their absorption bands influence on the pH chromophore absorbance measurements become insignificant.

This system can be readily adapted to portable spectrophotometers such as the commercially available Ocean Optics USB 2000 spectrometer (Denedin, FL). Further enhancements in sensitivity may be introduced by increasing the path length of light by using the thin fluoride sensing films in a wave-guide configuration [23]. The only limitation at present for integration with OPH/OPAA immobilized biosensors is the two-step reaction conditions required for hydrolysis and detection. When overcome, the sensing approach described here has the potential for creating highly selective and simple detection system capable of screening for the presence of a wide spectrum of OPs.

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Figure 1.
Schematic of the experimental set-up of the sensing film in cuvette

Figure 2.
Absorbance spectra of film on quartz slide treated with various concentrations of DFP hydrolyzed by OPH

Schematic 1.
Principle of detection