Acetylcholinesterase-Based Organophosphate Nerve Agent Sensing Photonic Crystal

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We developed a polymerized crystalline colloidal array (PCCA) photonic crystal sensing material that senses the organophosphorus compound parathion at ultratrace concentrations in aqueous solutions. A periodic array of colloidal particles is embedded in a hydrogel network with a lattice spacing such that it Bragg diffracts visible light. The molecular recognition agent for the sensor is the enzyme acetylcholinesterase (AChE), which binds organophosphorus compounds irreversibly, creating an anionic phosphonyl species. This charged species creates a Donnan potential, which swells the hydrogel network, which increases the embedded particle array lattice spacing and causes a red-shift in the wavelength of light diffracted. The magnitude of the diffraction red-shift is proportional to the amount of bound parathion. These AChE-PCCAs act as dosimeters for parathion since it irreversibly binds. Parathion concentrations as low as 4.26 fM are easily detected.

There is currently an urgent need for efficient, rapid detection of organophosphorus (OP) compounds. OP compounds are prominently used by the agricultural industry in the United States and worldwide in the form of pesticides and insecticides. These OPs have been detected in streams draining from urban watersheds and in agricultural watersheds.[1] OPs also occur in the form of chemical warfare nerve agents such as sarin, cyclosarin, VX, and tabun. These compounds are potent irreversible inhibitors of nervous system function[2,3] and are thus quite toxic.

At present, there are several techniques available for determining the concentration of OPs in solution. GC/MS and HPLC techniques are commonly used[4] and show detection limits in the nanomolar concentration range.[5] However, these techniques are time-consuming and require extensive sample preparation in order to detect environmentally significant levels of OPs. Field detection appears impractical.

There are also detection schemes that utilize flow injection methods in which acetylcholinesterase (AChE) is immobilized inside or upon a polymer. In these methods, the AChE is exposed to the OP, and then a solution is added containing a substrate and a reagent which react to form a chromophore. The chromophore absorbance determines the enzyme activity, which is decreased by binding of the OP. Leon-Gonzales and Townshend[6] achieved 8 nM detection limits for OPs using this technique.

Molecularly imprinted polymer (MIP) sol−gel films with high selectivity toward specific OP species are also used.[7] These MIPs utilize functionalized silanes as templates to selectively bind OPs. This OP binding has been coupled to fluorescence and electrochemical techniques to determine the OP concentration.

Leblanc et al.[8] have used a layer-by-layer deposition technique to make Langmuir−Blodgett multilayers of the enzymes acetylcholinesterase, organophosphorus hydrolase,[9,10] and organophosphorus acid anhydrolase.[10] They achieved nanomolar detection limits of the pesticide paraoxon[10,11] by monitoring fluorescence.

Amperometric detectors which sense OPs at nanomolar levels have also been fabricated. These detectors utilize AChE[12] or organophosphorus hydrolase[13,14] immobilized upon electrodes. Mulchandani et al.[13] achieved detection limits of 70 nM OP, while Sacks et al.[14] detected nanomolar concentrations of parathion. These techniques appear practical for field detection because the sensors are easily miniaturized and require compact, inexpensive instrumentation and require only modest sample preparation. However, these techniques may suffer interference from other oxidizable substances that may be present in real (field) samples.[14]

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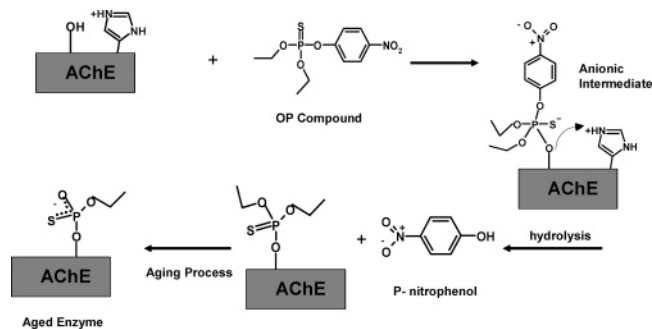
Figure 1. Phosphorylation of AChE by parathion. The result is a charged intermediate form, which on aging undergoes dealkylation to form a stable anionic phosphonyl adduct, which irreversibly inhibits AChE.
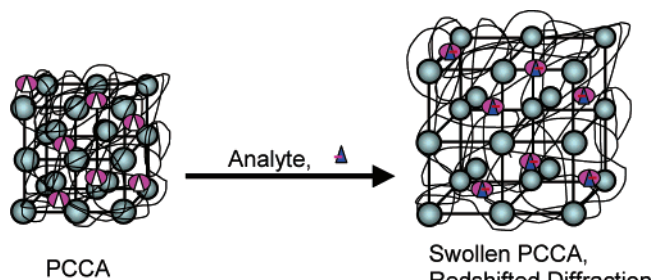

Figure 2. A CCA forming in an fcc structure due to electrostatic repulsion between particles. The particle spacings are set such that they diffract visible light. PCCAs are formed by polymerization of an acrylamide hydrogel network around the CCA. The PCCA backbone is functionalized with a molecular recognition agent, which binds the analyte, actuating either a shrinkage or a swelling of the hydrogel network. The embedded CCA lattice spacing changes as the volume changes, shifting the diffracted wavelength and the perceived color of the PCCA.
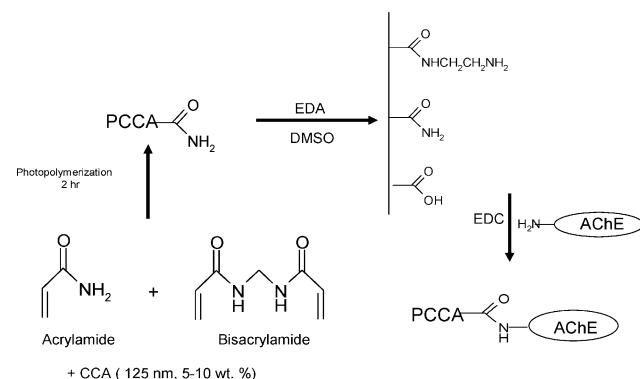
In the work here, we utilize AChE as the molecular recognition agent for parathion, an OP. AChE is an enzyme that functions at the neural synapse to hydrolyze the neurotransmitter acetylcholine to acetate and choline.[15] The hydrolysis depolarizes the nerve so that it can undergo another conduction event.

OPs inhibit AChE by irreversibly blocking substrate turnover. The OP binds to the active site serine, producing a phosphonyl–enzyme complex. When AChE is phosphorylated, it cannot function and the nerve remains polarized.[15] Figure 1 depicts the phosphorylation of AChE by parathion and its subsequent reactions. An anionic tetrahedral intermediate forms,[16,17] which dealkylates to form the "aged" product, which is a stable anionic phosphonyl adduct.[18] Millard et al. demonstrated using X-ray crystallography that the aged form of the phosphorylated enzyme involves a stable anionic adduct.[19]

Our OP sensor is based on our polymerized crystalline colloidal array (PCCA) photonic crystal sensing technology. These PCCAs utilize an array of colloidal particles[20–25] polymerized into an acrylamide hydrogel[26–29] that Bragg diffracts light in the visible spectral region (Figure 2). The molecular recognition group,

Figure 3. Preparation and functionalization of AChE-PCCA hydrogel backbone.

AChE, is covalently attached to the hydrogel to bind the analyte of interest and to actuate a volume increase proportional to the concentration of analyte. The hydrogel volume increase red-shifts the wavelength of light diffracted. Shifts in the wavelength of diffracted light are proportional to the analyte concentration. Our AChE PCCA sensor relies upon a volume phase transition of the hydrogel due to changes in the ionic free energy of the system.

The wavelength of light (λ_o) diffracted follows Bragg's law: $\lambda_o = 2nd \sin \theta$. In our AChE-PCCA, light is diffracted by the fcc 111 plane of the embedded array of particles. λ_o depends on the plane spacing, d, the refractive index of the system, n, and the incident angle of the light, θ, which is the Bragg glancing angle. Sin θ is unity since we are sampling back-diffraction of light normally incident to the 111 plane.

EXPERIMENTAL SECTION

PCCA Preparation. Figure 3 depicts the synthesis and functionalization of the PCCA. Acrylamide (0.10 g, 1.4 mmol, Fluka), N,N'-methylenebisacrylamide (2.5 mg, 16.2 μmol, Fluka), a colloid suspension (2.0 g, 5–10% w/w dispersion, polystyrene latex spheres, 110 nm diameter[20–25]) in Nanopure water (Barnstead), AG501-X8 (D) ion-exchange resin (~0.1 g, 20–50 mesh, mixed bed, Bio-Rad), and 10% diethoxyacetophenone (DEAP; 7.7 μL, 3.84 μmol; Aldrich) in DMSO (Fisher) were mixed in a 2-dr vial. The mixture, which was centrifuged to remove the ion-exchange resin, was injected between two quartz disks separated

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by a 125-μm-thick Parafilm spacer. The colloidal particles self-assemble into a CCA, giving rise to a liquid film that diffracts light. The film was exposed to 365-nm UV light from mercury lamps (Blak Ray) for 2 h. A polyacrylamide hydrogel network forms around the CCA, resulting in a polymerized CCA (PCCA).[26-29] The cell enclosing the PCCA was then opened in Nanopure water and the PCCA film allowed to equilibrate.

Chemical Modification of Hydrogel Backbone. The PCCA was transferred from 100% H_2O to 100% DMSO in 25% increments over the course of 1 h. The PCCA was then placed into 200 mL of 10% (v/v) solution of ethylenediamine (Fisher) in DMSO. This solution was heated to 90° C in a closed 500-mL round-bottom jacketed reaction vessel (Kontes). The reaction was allowed to proceed for 16 h. to convert the pendant amide groups to amines.[30] The PCCA was then removed and transferred stepwise back from DMSO to pure H_2O. After 2 h of washing in Nanopure water, the PCCA displayed a 574-nm diffraction maximum.

Attachment of Acetylcholinesterase to PCCA. A coupling solution of acetylcholinesterase (EC 3.1.1.7: V−S from electric eel, MW 260 000, 806 units/mg of solid, Sigma) was prepared by diluting 1.6 mg (2000 units) to a volume of 4 mL with 150 mM Tris buffer (Pierce Biotechnology) at pH 7.4. The PCCA was preincubated in the AChE solution for 48 h. The PCCA was removed from the AChE solution and dipped into a solution of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (0.032 g, 167 μmol, Pierce) in 2 mL of 150 mM Tris buffer (pH 7.4). The PCCA was then placed back in the AChE solution. Coupling was then allowed to proceed for 2 h, after which the PCCA was incubated for 48 h in 150 mM Tris to exchange out uncomplexed AChE.

The initial PCCA diffraction of 574 nm red-shifts to 636 nm due to attachment of AChE, presumably due to an increased free energy of mixing; AChE attachment results in an increased hydrogel hydrophilicity.

Functionality Test of AChE-PCCA. The sensor was tested for enzyme activity with acetylthiocholine iodide (ATChI, Sigma), a mimic of the natural substrate acetylcholine, using Ellman's acetylthiocholine hydrolysis assay.[31] ATChI was added to 100 mL of 10 μM 5,5′-dithiobis(2-nitrobenzoic acid) (Sigma) dissolved in 150 mM Tris nuffer (pH 8.0). ATChI concentrations were varied from 1 to 6 mM. The AChE-PCCA was then placed into the solution, and the UV−visible absorbance of the reaction solution was monitored against a 150 mM Tris buffer background. Absorbance at 412 nm was recorded over a 5-min time period at selected time intervals. The hydrolysis of ATChI in the absence of enzyme was also monitored.

Diffraction Measurements. The diffraction of the PCCA was monitored using a fiber-optic diode spectrometer with a tungsten halogen light source (Ocean Optics) using a reflectance probe. The high diffraction efficiency of the PCCA causes the light to be diffracted completely by the first 10−20-μm thickness of the PCCA.[25]

The diffraction was measured in aqueous solutions containing the OP pesticide parathion (Chem Service, Inc.). Stock solutions were made by dissolving 9.8 μL of parathion (0.0124 g, 42.6 μmol) in 2 mL of methanol (Fisher) and then diluting to 1 L with

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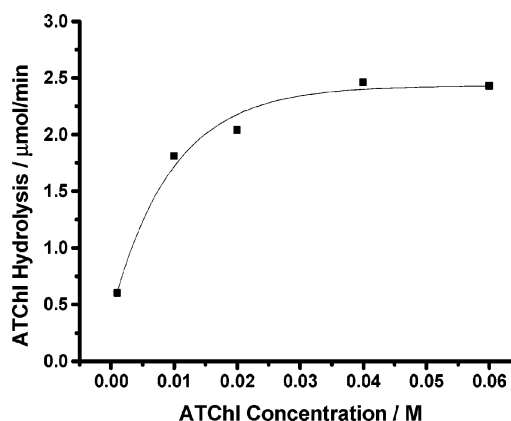(31) Ellman, G. L.; Courney, K. D.; Andres, V.; Featherstone, R. M. *Biochem. Pharmacol.* **1961**, *7*, 88−95.

Figure 4. Michaelis−Menten curve of Ellman's assay of AChE-PCCA confirming that some enzyme function is retained upon AChE attachment to PCCA backbone. The curve indicates that 2.4 units of active AChE are attached to the hydrogel backbone of the PCCA.

Nanopure water with vigorous stirring. A serial dilution was then done using Nanopure water. The parathion concentration ranged from 4.26 fM (4.26×10^{-15} M) to 42.6 μM (4.26×10^{-5} M). The pH was adjusted to ~7.0 using very small amounts of 0.1 M NaOH (Fisher). A buffer was not used in order to avoid attenuation of the Donnan potential by a high ionic strength. The AChE-PCCA was preequilibrated for 1 h in Nanopure water (pH 7.0) to allow it to reach equilibrium volume before exposure to parathion solutions.

The AChE-PCCAs were exposed to 100-mL sample solutions at each parathion concentration, beginning with 4.26 fM parathion, until the diffraction peak stopped shifting, indicating that the system has reached saturation. Lower concentrations required ~30 min. to stabilize. Diffraction spectra were recorded, and then the PCCA was rinsed with Nanopure water for 0.5 h. The postrinsing diffraction wavelength was recorded at each concentration level tested. In a control measurement, an aminoethylated PCCA without AChE attached was exposed to a parathion solution to ensure that the diffraction response requires the presence of AChE.

We also performed control experiments that mimicked the parathion measurements by preparing solutions identical to those described above, but in the absence of parathion. AChE-PCCAs were exposed to these samples for 0.5-h intervals, followed by 0.5-h rinsing with Nanopure water, after which diffraction spectra were recorded.

Another control experiment used a series of very dilute parathion solutions that had previously been measured. An AChE-PCCA that had never sensed parathion was exposed to the solutions to test the hypothesis that the sensor acts dosimetrically and that parathion was removed from solutions. In this case, the diffraction shift occurred only upon exposure to parathion solutions for which the previous sensor had saturated.

RESULTS AND DISCUSSION

Determination of Bound Acetylcholinesterase. We monitored the aqueous solution hydrolysis of ATChI solutions exposed to an AChE-PCCA by using the Ellman's assay in order to quantify the amount of AChE attached to the PCCA before exposure to parathion. The AChE-PCCA displays Michaelis−Menten enzyme kinetics for the hydrolysis of ATChI. The linear rate of reaction
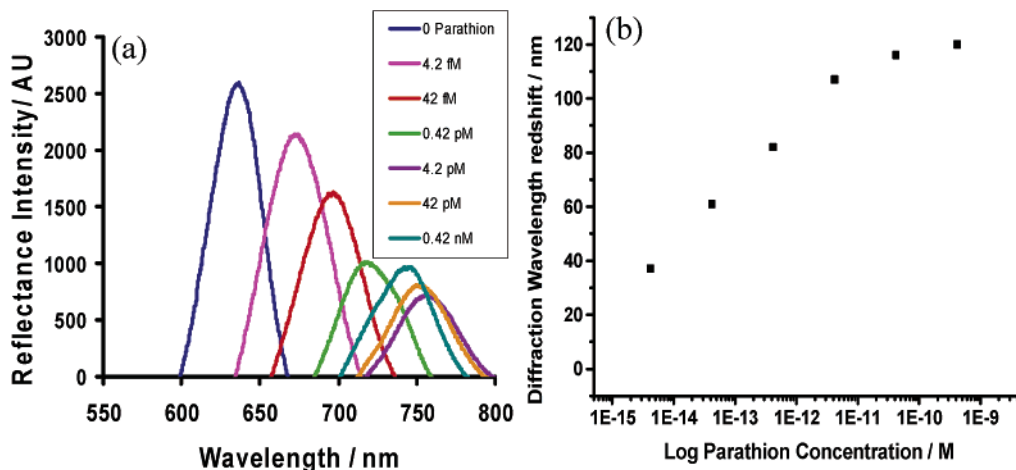
Figure 5. (a). Diffraction response of AChE-PCCA to 100-mL parathion solutions of varying concentrations. Saturation of response occurs at 42 pM parathion concentration. (b). Diffraction red-shift versus log of parathion concentration. AChE-PCCA displays saturation at 42 pM parathion solution exposure.

was calculated for AChE over the first 5 min for each trial. The reaction rates (μmol of ATChI/min) were then plotted against the substrate concentration to obtain a Michaelis–Menten plot (Figure 4). From the plot, we calculate a maximum rate of ATChI hydrolysis of 2.4 μmol/min. One unit of enzyme is the amount required to hydrolyze substrate at a rate of 1 μmol/min, which indicates the presence of 2.4 units, or at least 3.9×10^{-12} mol of active AChE attached to the gel. This calculation would underestimate the amount of attached enzyme if its activity were decreased by the immobilization. The natural hydrolysis rate in the absence of enzyme was measured to be 30 nmol/min averaged over 30 min. It appears that AChE retains a significant portion of its activity upon attachment to the hydrogel, probably because the reactive site is located at the bottom of a 20-Å-deep gorge.[32]

Response of AChE-PCCA to Parathion in Water. Figure 5 shows the diffraction response of an AChE-PCCA upon exposure to 100-mL solutions of parathion of different concentrations. The sensor displays a 37-nm diffraction shift after only 15 min in the presence of 100 mL of 4.2 fM parathion. Subsequent cycles of rinsing in Nanopure water and exposure to 100-mL solutions of increasing parathion concentrations yielded additional diffraction shifts until the diffraction response saturated (for a parathion concentration of ~42 pM) yielding a total spectral shift of ~120 nm.

For an infinite, irreversible binding constant, the amount of parathion that saturates our sensor would equal the number of moles of AChE attached to the PCCA. We calculate that we require 4.3×10^{-12} mol of parathion for saturation, which is similar to the amount of AChE in the PCCA (3.9×10^{-12} mol). The parathion-induced red-shift of the sensor is irreversible. Washing does not blue-shift the response.

To prove that parathion is complexed to the sensor, a series of subsaturation parathion solutions previously exposed to an AChE-PCCA sensor were exposed to a fresh AChE-PCCA. The hypothesis is that all of the parathion was removed by the first sensor. No significant change occurs in the diffraction wavelength of the second AChE-PCCA until the sensor is exposed to 42 pM

parathion. At this concentration, the first AChE-PCCA saturated, thus leaving some parathion in solution. Subsequent exposure of the second sensor to 0.42 and 4.2 nM parathion solutions completely saturates this second AChE-PCCA; all AChE sites become phosphorylated. The lack of response by the second sensor to the first four depleted solutions proves that parathion was removed by the first AChE-PCCA.

Response Mechanism. Three control experiments were performed to confirm that the diffraction response was due to the anionic species formed when the parathion inhibits the AChE. An aminoethylated, AChE-free PCCA was exposed to parathion solutions to prove that the diffraction shift was not due to interactions between the OP and the amine or carboxyl groups on the polymer backbone, but rather was due to the specific inactivation of the AChE's active site by the OP. No significant shift in the diffraction was observed.

If the response were due to formation of an anionic species that creates a Donnan potential, then no swelling would occur in a high ionic strength environment.[29] We see no response of the AChE-PCCA to parathion in the presence of 150 mM NaCl. Thus, the response is due to the formation of a covalently attached ionic species.

A final control measurement shows that the response at low parathion concentrations is not due to an impurity introduced during the sample preparations. We mimicked the preparation of parathion dilutions, but without using parathion. No diffraction shift occurs in the absence of parathion.

AChE-PCCA Is a Dosimetric Sensor for Organophosphates. Thus, our sensor mechanism is consistent with the expected mechanism in which parathion phosphorylates the serine residue of the AChE active site.[33] The tetrahedral adduct formed undergoes aging to form a stable anionic species.[17,18] Formation of an anionic charge on the phosphonyl sulfur group causes a Donnan potential inside the AChE-PCCA, which induces a negative osmotic pressure that draws water into the hydrogel matrix.[28,34–36] This forces the hydrogel to swell in low ionic

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strength solutions. The embedded CCA lattice spacing increases, causing a red-shift in the wavelength of Bragg-diffracted light.

CONCLUSIONS

We developed a novel material that can determine femtomolar concentrations of parathion in low ionic strength aqueous media. This detection limit is ~1 millionfold lower than other current detection methods. We are developing this sensor for use in detecting OPs in the field.

A transient sensing approach can be employed to overcome the high ionic strength interference from field samples.[37] The sensor would be exposed to field samples and then rinsed with deionized water. The OP would remain attached within the sensor while all other species would wash out. The diffraction wavelength shift of the rinsed sensor would be proportional to the amount of OP in the original sample.

The AChE sensor should be able to detect numerous OP species and could be used for visually monitoring OP levels in groundwater and air.

The sensor response window presently spans the red to near IR spectral region. We are working on increasing the spectral window so that it can span the entire visible spectral region.

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