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Communication—Microfluidic Electrochemical Acetylcholine Detection in the Presence of Chlorpyrifos

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An amperometric acetylcholine sensor was developed for use in a microfluidic system and characterized with chlorpyrifos and its metabolite, chlorpyrifos oxon. This enzymatic sensor was highly selective for acetylcholine, with a detection limit of 0.2 μM and sensitivity of 1.7 nA μM^{-1} from 1–150 μM. Though chlorpyrifos had no effect on sensor function, chlorpyrifos oxon significantly inhibited response across a range of concentrations (0.5–50 μM). Inhibition was reversed by 2-pyridine aldoxime methyl chloride. This platform can be used to both quantify acetylcholine in the presence of chlorpyrifos and as a biorecognition method for chlorpyrifos oxon.
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Organophosphates are used throughout the world as agricultural pesticides due to their high level of effectiveness.[1] According to the Environmental Protection Agency, between five and ten million pounds of the organophosphate (OP) chlorpyrifos (CPF) are used every year in the United States.[2] Recently, the states of California and New York have moved to institute a complete ban on chlorpyrifos based on epidemiological studies linking prenatal CPF exposure to neurobehavioral deficits in children.[3,4] At the same time, the consequences of CPF on human health are undergoing further evaluation to fully understand the extent of CPF's negative effects. To this end, an enzyme-based acetylcholine sensor that accurately detects acetycholine in the presence of CPF is presented for use in the investigation of OP toxicity.

Within the body, exposure to OP compounds leads to metabolic disruption and the alteration of acetylcholine levels. When neurons are functioning properly, acetylcholine—a neurotransmitter regulating processes from muscle contraction to learning and memory—is released into the neuronal synapse before being broken down by acetylcholinesterase.[5,6] OPs induce neurotoxicity through the inhibition of acetylcholinesterase, thus preventing the breakdown of acetylcholine. The subsequent increase in acetylcholine concentration leads to the overstimulation of the muscular, endocrine, and central nervous systems resulting in muscular fasciculation, decreasing motor activity, and respiratory depression.[7] OPs can also be quickly metabolized into their oxidized (oxon) forms, known to be more toxic and inhibiting acetylcholinesterase to a much higher degree.[8] Due to this effect of OPs on acetylcholinesterase, the inhibition of plasma and red blood cell cholinesterase is sometimes used as a quantification method for characterizing OP exposure, though it is only an indirect measure of acetylcholine concentration.[9] Therefore, the ability to directly detect and quantify changes in acetylcholine levels may assist in the early detection and treatment of OP poisoning.

The development of enzymatic biosensors for the electrochemical detection of acetylcholine can provide a low-cost but selective method for sample analysis. Other methods of detection, such as colorimetric and chromatographic techniques, provide high sensitivity but with greater time and expense required.[10–12] Some enzymatic sensors, like the one presented here, can be coupled with microfluidic platforms—such as the microclinical analyzer (μCA)—increasing throughput and providing the ability to automate the calibration and analysis process.[13] These full analysis platforms, with customizable flow rates and sample times, can provide new insight into the effects that organophosphates may have on acetylcholine metabolism.

In this work, an enzymatic acetylcholine sensor was developed for use with the μCA electrochemical detection platform. Detection parameters of the sensor toward acetylcholine were established both with and without CPF. The μCA was used to expose the sensor to chlorpyrifos oxon (CPO)—a primary metabolite of CPF—at various flow rates and exposure times to study changes in sensor inhibition. Sensors inhibited by CPO were regenerated though treatment with 2-pyridine aldoxime methyl chloride (2-PAM),[14] an antidote for OP poisoning. This electrochemical microfluidic platform accurately detected acetylcholine in the presence of CPF and can be used as a tool for the biorecognition of CPO through sensor inhibition and regeneration.

Experimental

Acetylcholine sensor fabrication and incorporation into the μCA.—The largest band electrode on a screen-printed electrode array (Pine Research, Durham, NC) was used as a Ag/AgCl quasi-reference, while three disk electrodes were made to be selective for acetylcholine (acetylcholine chloride, Sigma Aldrich, St. Louis, MO) using acetylcholinesterase from *Electrophorus electricus* (137 Units/mg, Sigma), and choline oxidase from *Alcaligenes* (15 Units/mg, Sigma). Each enzyme was dissolved separately [10 mg/mL, 50 mM potassium monobasic-sodium buffer at pH 7 (Fisher Scientific, Hampton, NH)] with bovine serum albumin (800 mg/mL, Sigma) and stored until use (−18°C). When required, these enzymes were combined equally (v/v), mixed with glutaraldehyde (0.5% wt/v, Sigma), vortexed (∼5 sec), drop-cast (1 μL) onto each working electrode, and air-dried (1 hour) before either use or storage [low light, 4°C, buffer solution (2mM, pH 7), 120 mM KCl]. To incorporate these sensors into the μCA, they were sealed within the housing and attached to a microformulator (Vanderbilt Institute for Integrative Biosystems Research and Education)—a microfluidic pump and valve system providing automated sensor calibration and sample analysis.[13]

Characterization of acetylcholine sensor.—The limits of detection (LOD) and quantitation (LOQ), linear range, V_{max}, and K_m for the acetylcholine sensor were determined using the μCA as they were for other enzyme electrodes.[13,15] Calibrations were performed by monitoring the current generated by calibrants (1 μM to 1 mM acetylcholine) with and without 50 μM CPF in buffer. Buffer for all experiments was made from a 50 mM potassium monobasic-sodium buffer (pH 7.0, Fisher) and diluted in water with KCl to produce a 2 mM phosphate, 120 mM KCl, pH 7 buffer solution. Current was monitored using a CHI 1440 potentiostat (CH Instruments, Austin, TX) held at 0.6 V vs. Ag/AgCl. Calibrants were sampled using a microformulator (100 μL/min.) pump and valve system. Linear regressions were performed on the linear ranges of the calibration data. The resulting slopes provided the sensitivity of the electrode with and without 50 μM CPF present. The limits of detection (LOD) were calculated according to Equation 1,

$$LOD = \frac{3S_E}{m} \quad [1]$$

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where S_E is the error of the y-intercept, and m is the slope of the calibration. The equation used for calculating the limit of quantitation is similar, except that the standard error was multiplied by ten instead of three. Because the enzyme saturation followed Michaelis-Menten kinetics, a hyperbolic function could be fit to the data to determine V_{max} and K_m. The resulting equation of the hyperbola provided coefficients corresponding to the values for V_{max} and K_m. All p-values calculated in this study were found by performing t-tests assuming unequal variance. Stability of the sensor was tested by exposing the sensor to 50 μM acetylcholine every hour for 12 hours and measuring the changes in current response. Following the 12-hr experiment, the sensor was stored in buffer solution (4°C, low light). This sensor was tested again both one and two weeks later to determine the effects of storage on sensor response.

Sensor inhibition using CPF and CPO.—To test the effect of CPF and CPO on acetylcholine detection, the sensor response to acetylcholine was measured both before and after exposure to either compound. For initial acetylcholine measurements, the sensor was first allowed to reach steady-state current in buffer (10 min.), before being treated with acetylcholine (50 μM, 5 measurements, 2 min. each). After each acetylcholine measurement, buffer was run over the sensor (2 min.) to return to baseline (100 μL/min.). The sensor was then exposed to an OP solution [0.5 – 50 μM CPF or CPO (Sigma), 100 – 500 μL/min., 10 – 30 min.] to determine how flow rate, OP concentration, and exposure time effected sensor inhibition. Each inhibition was followed by a ten-minute buffer wash period before acetylcholine measurements were taken again. Inhibition percentage was calculated using Equation 2, where $I\%$ is the inhibition percentage, i_0 is the steady-state current generated by acetylcholine before OP exposure, and i_1 is the steady-state current generated by acetylcholine after OP exposure. The current values used for i_0 and i_1 are averages of the currents generated by five acetylcholine measurements before and after OP exposure.

$$I\% = 100 \times \left(\frac{i_0 - i_1}{i_0}\right) \quad [2]$$

Sensor regeneration.—Sensor regeneration was investigated by using 2-PAM to counteract the effects of CPO. After reaching steady state, the current generated by acetylcholine (50 μM) was measured five separate times alternating with buffer to return to baseline. The sensor was then exposed to CPO (5 μM, 100 μL/min., 30 min.) and the response to acetylcholine was measured as before. The inhibition percentage was again calculated as in Equation 2. Sensors were regenerated by 2-PAM (500 μM, in buffer, 30 min., Sigma), followed by another set of acetylcholine measurements. Regeneration percentage was determined using Equation 3, where $R\%$ is the regeneration percentage, i_2 is the steady-state current generated by acetylcholine after 2-PAM treatment, and i_0, as before, is the steady-state current generated by acetylcholine before OP exposure.

$$R\% = 100 \times \left(\frac{i_2}{i_0}\right) \quad [3]$$

Results and Discussion

The need to analyze systems directly affected by CPF necessitates the development of an acetylcholine sensor that can function in the presence of CPF. Consequently, it is essential to understand how CPF changes the activity of the sensor. Some studies have used acetylcholinesterase in colorimetric or nanoparticle-based assays to detect CPF, as the competitive inhibition of acetylcholinesterase by CPF results in inactivation of the enzyme.[16,17] One way to explore the effects of CPF on enzyme activity is through kinetics measurements like V_{max} and K_m—measurements of maximum enzyme rate and concentration of substrate at half V_{max}, respectively. Occupation of acetylcholinesterase active sites by CPF will result in lower V_{max} and K_m values. As shown in Fig. 1 (left), both V_{max} and K_m are nearly identical for acetylcholine with and without 50 μM CPF. V_{max} values

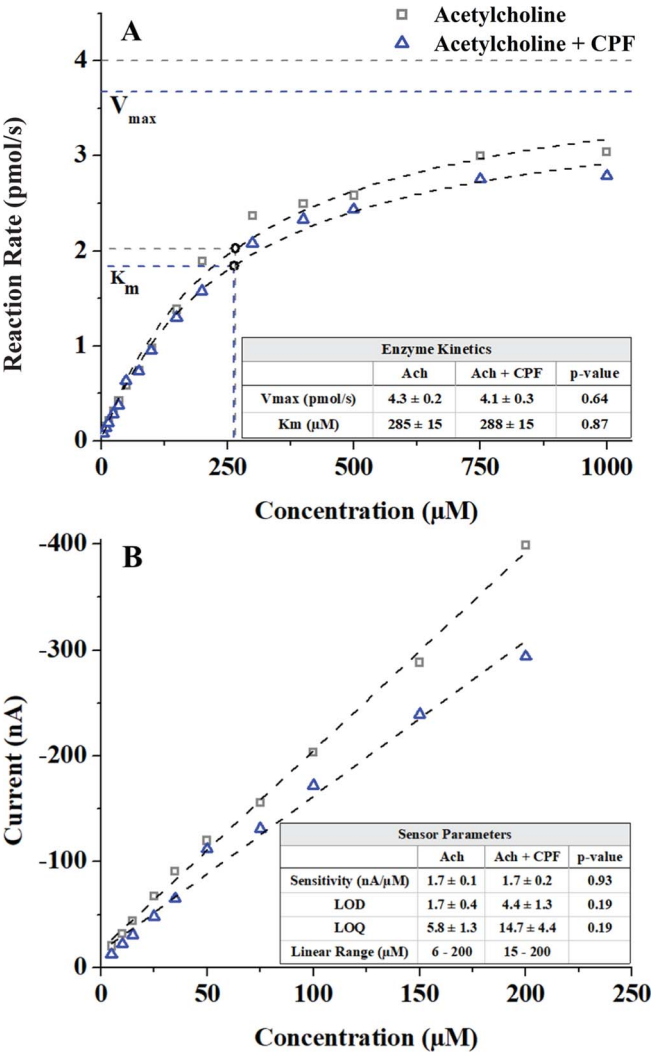

Figure 1. Representative reaction rate vs. concentration (left) and current vs. concentration (right) graphs for acetylcholine (gray squares) and acetylcholine with 50 μM CPF (blue triangles). Calibrants were sampled through a microformulator (1 μM to 1 mM acetylcholine, 2 min.) with buffer in between (2 min.) at 100 μL/min., 25°C. A) Calibration showing enzyme saturation for each set of solutions. Table: Enzyme kinetics, V_{max} and K_m, for each solution set. B) Scatterplot of the linear range for each solution set. Table: Detection parameters of the sensor including LOD, LOQ, linear range, and sensitivity with and without CPF. For both enzyme kinetics and sensor parameters, significance testing (t-test assuming unequal variance, n = 3) was performed across solution sets.

of 4.3 ± 0.2 and 4.1 ± 0.3, for acetylcholine and CPF solutions respectively, and K_m values of 285 ± 15 and 288 ± 15 result in p-values of 0.64 for V_{max} and 0.87 for K_m signifying no significant difference in enzyme kinetics with the addition of CPF. These results indicate that the enzyme's active sites are not being populated by CPF but are instead available to cleave acetylcholine.

These conclusions are further supported by the sensor response metrics. In the absence of CPF, the acetylcholine sensor's linear range was 1–150 μM, with low detection and quantitation limits (0.2 ± 0.1 μM and 0.7 ± 0.1 μM respectively) and high sensitivity (1.7 ± 0.1 nA μM^{-1}, Fig. 1, right). The sensor also demonstrated good operational stability, retaining 82 ± 3% of its response over 12 hours of continuous use and 59 ± 6% of the response after two weeks in storage. Including 50 μM CPF in the calibrant solutions had minimal effects on these values, with similarly low detection and quantitation limits (0.8 ± 0.2 μM and 2.5 ± 0.6 μM respectively, p-values of 0.09 compared to no CPF), along with a sensitivity and linear range comparable

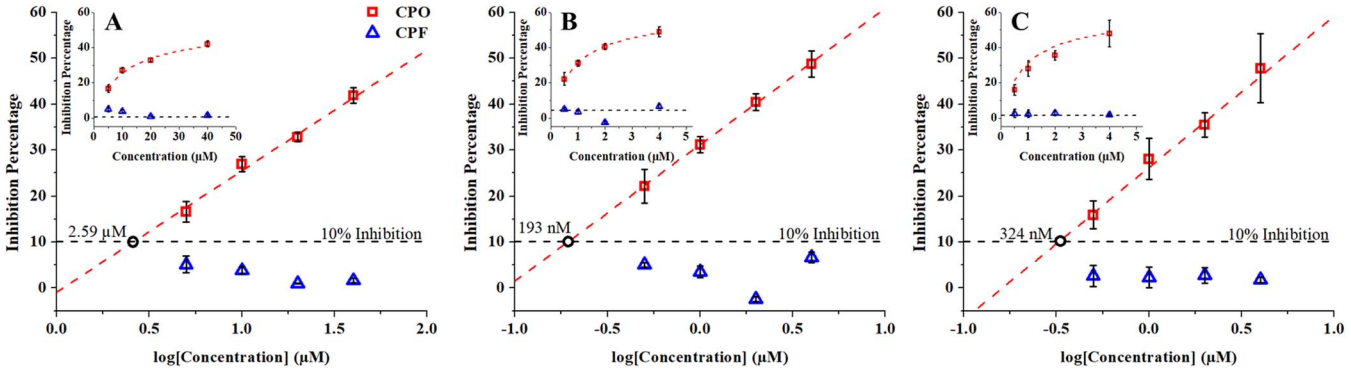

Figure 2. Sensor response to CPO (red squares) and CPF (blue triangles) at multiple flow rates and treatment times: A) 100 μL/min. for 10 min. B) 100 μL/min. for 30 min. and C) 500 μL/min. for 10 min. **Insets**: Inhibition percentage vs. [CPO/CPF] showing a logarithmic increase in inhibition with increasing CPO concentration and no substantial inhibition from CPF compared to controls (dotted line). **Graphs**: Inhibition percentage vs. log[CPO/CPF] resulting in a linear relationship for CPO inhibition as expected for Michaelis-Menten kinetics. The 10% inhibition concentration decreased with increasing flow rate and treatment time, reaching as low as 193 nM at 100 μL/min. for 30 min. Experiments were performed in the μCA with acetylcholine (50 μM, 2 min., 100 μL/min.) and CPO/CPF (0.5 – 40 μM) with treatments of 10 to 30 min. at flow rates of 100 to 500 μL/min. Data represented as mean and SE (n = 3).

to the CPF-free solutions (1.7 ± 0.2 nA μM^{-1}, p-value = 0.93, 3–150 μM). Over the course of this experiment, the acetylcholine sensor was treated with 50 μM CPF—175 times higher than the Drinking Water Equivalence Level—for well over 30 minutes, with negligible effects on sensor function.[18] As a result, this sensor could be useful in analyzing changes in acetylcholine concentration in systems containing high levels of CPF.

To further characterize sensor performance, the microformulator—an automated pump and valve system—was used to adjust exposure times and flow rates to establish the effects of these variables on sensor function. At every flow rate and exposure time tested, CPF did not inhibit sensor function, with any losses instead being comparable to the signal decrease seen in the controls. Conversely, CPO-induced enzyme inhibition consistently followed Michaelis-Menten kinetics (Fig. 2, inset), enabling the creation of linear calibration curves that allow for CPO quantification (Fig. 2). Using 10% as a standard for sensor inhibition, a flow rate of 100 μL/min and a exposure time of 10 minutes resulted in a calculated minimum detectable concentration of 3 μM CPO. This minimum inhibition point of 10% was well above typical signal losses due to time-dependent decreases in enzyme activity. Even with a 10% inhibition standard, the detection limit went as low as 193 nM by increasing exposure time to 30 min. Similarly, increasing the flow rate during CPO exposure from 100 to 500 μL/min. resulted in 10% sensor inhibition with 324 nM CPO for 10 minutes. While other studies have described sensors utilizing acetylcholinesterase for OP detection, the μCA microfluidic system allows for the variation in flow rate and exposure time, resulting in the biorecognition of CPO by the acetylcholine sensor at concentrations as low as 193 nM.[19–22]

The regeneration of a CPO-inhibited acetylcholine sensor by 2-PAM introduces the opportunity for rapid quantification of CPO and other highly toxic OPs. At the sensor surface, acetylcholinesterase inhibition occurs when OP compounds bind to active site serines, preventing the enzyme from cleaving acetylcholine. This bond between the OP and acetylcholinesterase is reversible for only a short period of time before an aging process occurs that renders it irreversible.[23] To demonstrate this reversibility, the sensor underwent regeneration treatments using 2-PAM (a treatment for OP poisoning) to reactivate the acetylcholinesterase used in sensor fabrication.[24,25] During these experiments, the microformulator pump and valve system was used to first inhibit the sensor using CPO (5 μM, 30 min.) before regenerating with 2-PAM (500 μM, 30 min.), and measuring the response to acetylcholine (50 μM) after inhibition and again after regeneration. Following CPO exposure, the signal decreased to an average of 26 ± 1% of the original signal (Fig. 3). Subsequent treatment with 2-PAM resulted in regaining 78 ± 4% of the pre-inhibition signal. Though the regeneration percentage remained consistent, by the fifth regeneration attempt only 29 ± 7% of the original signal remained. Even so, the ability to regenerate the sensor after OP inhibition can substantially increase sensor reusability, decreasing both time and cost associated with using this sensor as a quantification method.

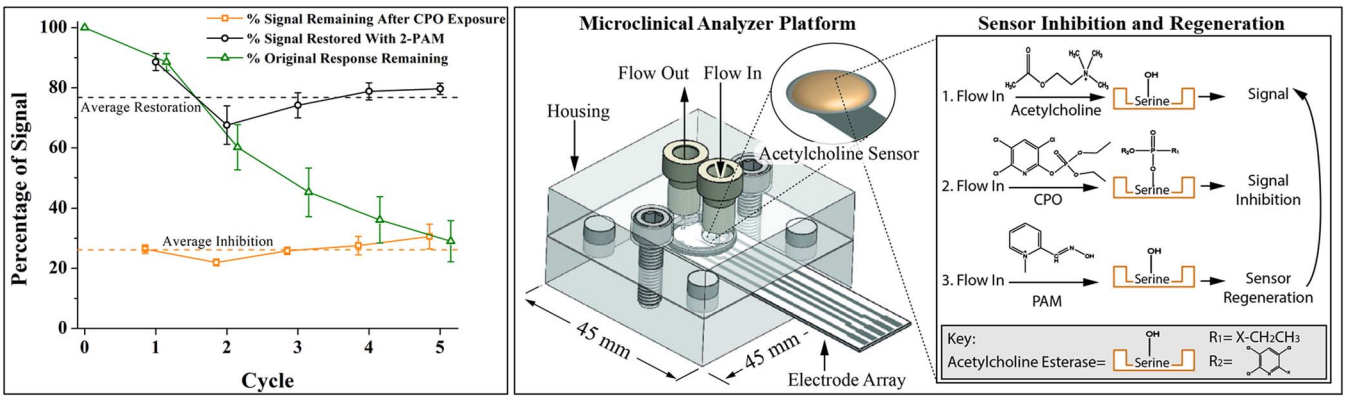

Figure 3. Summary of five inhibition and regeneration cycles using the μCA electrochemical detection platform. **Left**) After inhibition, an average of 26 ± 1% of the previous signal remained (orange dashed line). Following regeneration, an average of 78 ± 4% of the lost signal was regained (black dashed line). This repetitive regeneration resulted in 29 ± 7% of the original signal remaining after the fifth cycle (green triangle, cycle 5). **Right**) Drawing of the μCA electrochemical detection platform with the sensor inhibition and regeneration workflow. All experiments were conducted within the μCA using acetylcholine (50 μM, 2 min.), CPO (5 μM, 30 min.) and 2-PAM (500 μM, 2 mM PBS, 120 mM KCl, 30 min.). Data represented as mean and SE (n = 3).

Summary

OP exposure, common among those working with agricultural pesticides, directly and irreparably alters neural function if not properly treated. Here, the effects of OPs on a newly developed acetylcholine sensor were studied. Enzyme kinetics and sensor parameters were not significantly different between calibrations using acetylcholine either with or without 50 μM CPF, and as such the sensor could be used to accurately quantify acetylcholine in the presence of high quantities of CPF (<50 μM CPF). The μCA was used to provide flexibility in experimental design and expose the sensor to CPO at various flow rates and exposure times. CPO quantification and 2-PAM regeneration showed that multiple inhibition and regeneration events are possible. This sensor platform can provide insights into the effects of OPs on acetylcholine in biological systems exposed to CPF, while also serving as a platform for the detection of the most toxic OPs.

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