

Poly(ethylene glycol) Hydrogel-Encapsulated Fluorophore–Enzyme Conjugates for Direct Detection of Organophosphorus Neurotoxins

Ryan J. Russell and Michael V. Pishko*

Department of Chemical Engineering, Texas A&M University, College Station, Texas 77843-3122

Aleksandr L. Simonian and James R. Wild

Department of Biochemistry and Biophysics, Texas A&M University, College Station, Texas 77843-2128

A simple approach is described for preparing poly-(ethylene glycol) hydrogel materials with encapsulated seminaphthofluorescein (SNAFL)–organophosphorus hydrolase enzyme conjugates. Direct determination of enzyme-catalyzed neurotoxin hydrolysis is provided by the self-referencing, pH-sensitive dye SNAFL-1, whose emission spectrum changes at $\lambda = 550$ in response to pH. Using spectrofluorimetry and paraoxon as a model organophosphate, paraoxon concentrations as low as 8×10^{-7} M could be readily detected. On the basis of the signal-to-noise ratio, a detection limit of 16 nM was determined. The materials demonstrated high stability against enzyme-denaturing, leaching, and photobleaching when stored under ambient conditions.

Organophosphorus hydrolase (OPH) is an enzyme that exhibits the unique ability to hydrolyze a large variety of organophosphate pesticides and neurotoxins including paraoxon, parathion, acephate, Sarin, and VX.¹ OPH selectively catalyzes a hydrolytic reaction in the P–O, P–S, P–F, or P–CN bonds in neurotoxins, resulting in the stoichiometric generation of two protons. Direct neurotoxin detection is thus possible via measurement of the pH change associated with enzyme activity.² Recombinant OPH enzymes are stable, are highly active, and have been used in chemical warfare agent degrading foams without loss of activity.³ Mutant enzymes which exhibit altered substrate specificity have been produced by amino acid substitution; these mutations selectively improve the ability of OPH enzymes to hydrolyze specific agents (e.g., Soman, acephate, or VX).^{4,5}

Current neurotoxin sensors have primarily focused upon an indirect measure resulting from the inhibition of acetylcholinesterase (AChE). This approach provides high sensitivity by deter-

mining the “total anticholinesterase activity” of samples. While sensitive, current approaches are limited in specificity because cholinesterases are the target of a wide variety of toxic inhibitors, from heavy metals to chemical warfare agents. Widely used carbamate pesticides also inhibit AChE,⁶ while OPH exhibits high selectivity against carbamates.⁷ Additionally, most sensors developed using AChE inhibition have lengthy response times due to long incubation periods, and the inhibition is irreversible without subsequent enzyme-reactivation steps. Early approaches to fluorescence-based detection schemes demonstrated sensitivity to millimolar concentrations, but highly nonlinear behavior toward micro- and nanomolar concentrations.^{8,9} Both sensors were based upon fluorescein quenching and thus susceptible to changes in light intensity, photobleaching, and nonspecific quenching. While two recent additions to the literature have used optical techniques and OPH to experimentally measure paraoxon concentrations as low as 0.01 mM with detection limits extrapolated as low as 2 μ M,^{10,11} detection limits for amperometric-based OPH sensors have recently been extended as low as 90 nM.¹²

In this paper, we report the development of a fluorescent biosensor for OP neurotoxins utilizing carboxy seminaphthofluorescein (SNAFL-1), a ratiometric pH-dependent fluorophore, incorporated with native OPH into a poly(ethylene glycol) (PEG) hydrogel. Recently Rogers and co-workers adsorbed fluorescein isothiocyanate–OPH conjugates onto the surface of poly(methyl methacrylate) beads.¹¹ Here we have immobilized OPH within a PEG hydrogel, which should provide a higher density of OPH than can be achieved via surface modification. In addition, the PEG gel provides a protective environment for the enzyme and inhibits degradation and fouling. These hydrogels have a high equilibrium water content and thus are highly swollen in aqueous

* Corresponding author. Tel.: (409) 847-9395. Fax: (409) 845-6446. E-mail: pishko@tamu.edu.

- (1) Grimsley, J. K.; Scholtz, J. M.; Pace, C. N.; Wild, J. R. *Biochemistry* **1997**, *36*, 14366–14374.
- (2) Simonian, A. L.; Rainina, E. I.; Wild, J. R. *Anal. Lett.* **1997**, *30*, 2453–2468.
- (3) LeJeune, K. E.; Swers, J. S.; Hetro, A. D.; Donahey, G. P.; Russell, A. J. *Biotechnol. Bioeng.* **1999**, *64*, 250–254.
- (4) diSioudi, B.; Grimsley, J. K.; Lai, K.; Wild, J. *Biochemistry* **1999**, *38*, 2866–2872.
- (5) Grimsley, J. K.; di Sioudi, B.; Wild, J. R. *Biotech. Intl.* **1999**, *11*, 235–242.

- (6) Skladal, P. *Food Technol. Biotechnol.* **1996**, *34*, 43–49.
- (7) Mulchandani, A.; Kaneva, I.; Chen, W. *Anal. Chem.* **1998**, *70*, 5042–5046.
- (8) Rogers, K. R.; Cao, C. J.; Valdes, J. J.; Eldefrawi, A. T.; Eldefrawi, M. E. *Fundam. Appl. Toxicol.* **1991**, *16*, 810–820.
- (9) Bronk, K. S.; Michael, K. L.; Pantano, P.; Walt, D. R. *Anal. Chem.* **1995**, *67*, 2750–2757.
- (10) Mulchandani, A.; Pan, S.; Chen, W. *Biotechnol. Prog.* **1999**, *15*, 130–134.
- (11) Rogers, K.; Wang, Y.; Mulchandani, A.; Mulchandani, P.; Chen, W. *Biotechnol. Prog.* **1999**, *15*, 517–521.
- (12) Mulchandani, A.; Mulchandani, P.; Chen, W.; Wang, J.; Chen, L. *Anal. Chem.* **1999**, *71*, 2246–2249.

environments. The photopolymerized PEG hydrogel serves to both stabilize and immobilize the fluorescent dye–enzyme conjugate. These hydrogels were chosen as the sensor material because they were previously shown to be nonfouling in complex environments.¹³ PEG-enzyme modifications also frequently result in increased enzyme stability without a significant loss in activity.¹⁴ Within the hydrogel, immobilized OPH catalyzes the hydrolysis of organophosphate neurotoxins, thus generating protons, which result in a decrease in the microenvironment pH and an increase in the measured fluorescence intensity. SNAFL-1 is a self-referencing dye, which reduces problems associated with photobleaching and decreases sensor noise through the use of ratiometric emission measurements.

Recently, a bead-based optical array was reported which utilized fluorescent microspheres to simulate an artificial nose.¹⁵ OPH immobilization into this hydrogel provides the first step toward development of a fiber optic or bead-based optical array which can utilize numerous mutant forms of genetically engineered OPH enzymes with different substrate specificity to identify a wide variety of neurotoxins. The hydrogel microspheres described herein are ideally suited for such a sensor system.

EXPERIMENTAL SECTION

Reagents. Wild-type OPH (E. C. 3.1.8.1) was isolated from a recombinant *Escherichia coli* strain using a published procedure.⁴ Potassium phosphate monobasic, sodium phosphate dibasic heptahydrate, sodium chloride, and diethyl *p*-nitrophenyl phosphate (paraoxon) were obtained from Sigma Chemical Co. (St. Louis, MO). Poly(ethylene glycol) diacrylate with a molecular weight of 575 (PEG-DA), trimethylolpropane triacrylate (TPT), and 2,2 dimethoxy-2-phenylacetophenone (DMPA) were obtained from the Aldrich Chemical Co. (Milwaukee, WI). Dimethacrylate PEG (MW 1000) and tetrahydroxy PEG (MW 18 500) were obtained from Polysciences (Warrington, PA). Light paraffin oil and *n*-heptane were purchased from Fisher Scientific, Inc (Pittsburgh, PA). The α -acryloyl, ω -*N*-hydroxysuccinimidyl ester of poly(ethylene glycol)-propionic acid, MW 3400 (PEG NHS-3400), was purchased from Shearwater Polymers, Inc (Huntsville, AL). All reagents were used as received. One hundred millimolar phosphate-buffered saline (PBS) consisted of 1.1 mM potassium phosphate monobasic, 3 mM sodium phosphate dibasic heptahydrate, and 150 mM NaCl in 18 M Ω ·cm deionized water (E-pure, Barnstead).

Preparation of SNAFL-OPH Conjugate. A 1 mg/mL stock solution of enzyme was prepared in 100 mM phosphate-buffered saline (PBS, pH 8.2). SNAFL-conjugated organophosphorus hydrolase (SNAFL-OPH) was prepared by reacting 1 mg of SNAFL-1 succinimidyl ester (Molecular Probes, Eugene, OR) dissolved in 100 μ L of DMSO with the lysine residues present on wild-type OPH, similar to other established succinimidyl ester protocols.¹⁶ OPH was reacted with the fluorophore for 1 h at pH 8.5.

Characterization of SNAFL-OPH. The enzymatic behavior of the fluorophore-labeled enzyme with paraoxon was characterized in an aqueous environment using a QM-1 fluorescence

spectrometer (Photon Technologies International, Monmouth, NJ) and an excitation wavelength of 510 nm. The pH-induced changes in SNAFL emission intensity were monitored at 550 and 620 nm. Fifty microliters of SNAFL-OPH (\sim 1 mg/mL) were dissolved in 3 mL of 1 mM PBS (pH 7.93, 150 mM NaCl), and the enzymatic activity was monitored by titration with 2.5 mM paraoxon while the solution was continuously stirred. Additionally, a control experiment was performed over multiple pHs in the absence of OPH enzyme to verify there was no production of *p*-nitrophenol or significant changes in pH. PEG-encapsulated enzymes were similarly characterized.

Preparation and Analysis of PEG-immobilized SNAFL-OPH. The dye-conjugated enzyme was immobilized in UV-photopolymerized PEG hydrogels constructed similarly to those reported earlier.¹⁷ SNAFL-OPH was acrylated by reaction with the α -acryloyl, ω -*N*-hydroxysuccinimidyl ester of PEG-propionic acid and then photopolymerized from a precursor solution which consisted of 50 μ L of SNAFL-OPH; 100 μ L of TPT; 10 mg of DMPA; and 1.5 mL of a multifunctional, acrylated or methacrylated PEG polymer. Microspheres were constructed from PEG macromers of either PEG diacrylate (MW 575), PEG tetraacrylate (MW 18 500),¹⁸ or PEG dimethacrylate (MW 1000). Tetraacrylated PEG was prepared by acrylation of tetrahydroxy PEG according to a published protocol.¹⁸ PEG 18 500 precursor solutions were prepared from 60% (w/v) PEG in PBS. Precursor solutions of PEG dimethacrylate were prepared by melting the polymer at 37 $^{\circ}$ C and mixing the components while incubating in an isothermal water bath at 37 $^{\circ}$ C. Spheres were swelled in 1 mM PBS to their equilibrium water content overnight prior to fluorescent analysis.

RESULTS AND DISCUSSION

As described in detail in the following sections, we fabricated PEG hydrogels containing covalently immobilized SNAFL-OPH. These fluorescent gels were then exposed to paraoxon, a model organophosphate neurotoxin, and characterized spectroscopically to determine the ability of the gel to sense changes in paraoxon concentration.

Fluorescent Characterization of SNAFL-OPH in Aqueous Solution. The fluorescent behavior of SNAFL-OPH in buffered aqueous solution was examined initially to characterize the excitation/emission spectral properties of the conjugated dye, verify the fluorophore–enzyme conjugate would be responsive to pH-induced changes from the hydrolysis of paraoxon, and characterize this response in the absence of any cofounders potentially introduced by the polymer encapsulation, such as denaturation of enzyme, mass transport limitations, or fluorescence quenching.

Unbound SNAFL-1 in 1 mM PBS (pH 7.2) had excitation/emission maximums at \sim 510/541 nm. Similar to published reports of SNAFL-based pH probes,¹⁹ the dye emission spectra experienced a redshift in the acid peak upon conjugation to OPH. The emission maximum after conjugation was 550 nm. The dye-

(13) Sheth, S.; Leckband, D. *Proc. Natl. Acad. Sci. U.S.A.* **1997**, *94*, 9399–8404.

(14) Delgado, C.; Francis, G.; Fisher, D. *Crit. Rev. Ther. Drug Carrier Syst.* **1992**, *9*, 249–304.

(15) Dickinson, T.; Michael, K.; Kauer, J. S.; Walt, D. R. *Anal. Chem.* **1999**, *71*, 2192–2198.

(16) Brinkley, M. *Bioconjugate Chem.* **1992**, *3*, 2–13.

(17) Russell, R.; Pishko, M.; Gefrides, C.; McShane, M.; Cote, G. *Anal. Chem.* **1999**, *71*, 3126–3132.

(18) Pathak, C.; Sawhney, A.; Hubbell, J. J. *Am. Chem. Soc.* **1992**, *114*, 8311–8312.

(19) Xuy, Z.; Rollings, A.; Alcalá, R.; Marchant, R. E. *J. Biomed. Mater. Res.* **1998**, *39*, 9–15.

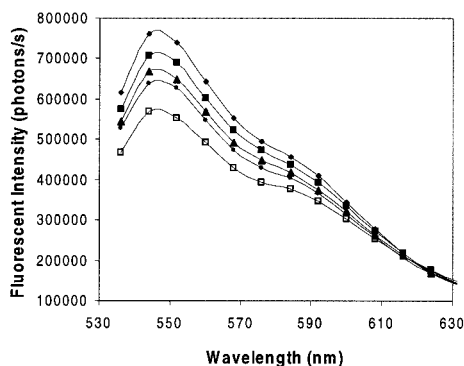


Figure 1. Fluorescence spectra of SNAFL-labeled organophosphorus hydrolase (16.7 $\mu\text{g/mL}$) upon exposure to paraoxon in 1 mM phosphate buffered saline (150 mM NaCl; pH 7.93; \square 0, \bullet 0.4, \blacktriangle 2.3, \blacksquare 4.3, \blacklozenge 10.2 μM of paraoxon).

conjugated enzyme was only lightly labeled, similar to results reported by Rogers et al. for FITC labeling of OPH.¹¹

OPH hydrolysis of paraoxon resulted in the release of protons, production of *p*-nitrophenol and diethyl phosphate, and a corresponding decrease in solution pH, as indicated by the change in fluorescence intensity (Figure 1). Under constant stirring, the fluorescent response was extremely rapid (<45 s), with larger concentrations of paraoxon initially overshooting the steady-state fluorescence. Ratiometric analysis of the SNAFL-1 fluorescent acidic and basic emission peaks revealed a linear increase in relative fluorescence for concentrations of paraoxon between 0.4 and 10 μM and positive but nonlinear correlation above 35 μM of paraoxon, similar to earlier biosensor results using electrochemical pH probes.²⁰ Additionally, the buffered solution acquired an increasingly yellow tint from the increasing concentration of *p*-nitrophenol, indicative that the enzyme was catalyzing the hydrolysis of paraoxon. A control experiment performed without enzyme, using only unconjugated SNAFL-1 and paraoxon over a range of pHs (7.0–11.5) did not result in production of *p*-nitrophenol (determined by lack of color formation) or demonstrate significant changes in pH. Studies with different pH buffers indicated that the fluorescent sensitivity to paraoxon can be tailored to specific concentration ranges by changing the initial microenvironment pH (data not shown), which can be easily accomplished in the hydrogels by simply exchanging the surrounding solution.

Fluorescent Characterization of PEG-Encapsulated SNAFL-OPH. The fluorescent response of polymer-immobilized SNAFL-OPH exhibited some differences from that found in aqueous solution. An acidic shift in the reported SNAFL-1 pK_a due to the hydrogel microenvironment pH was noticed for all three types of PEG macromers, a result consistent with prior microenvironment pH differences observed when immobilizing enzymes into hydrogels such as poly(2-hydroxyethyl methacrylate).²¹ The acrylated PEG hydrogels (MW 575 and 18 500) both showed an internal pH shift in the hydrogel-immobilized dye of nearly 3.5 pH units in 1 mM PBS buffer. Shifts as large as 4.5 pH units have previously been reported for enzymes immobilized on polymer supports in

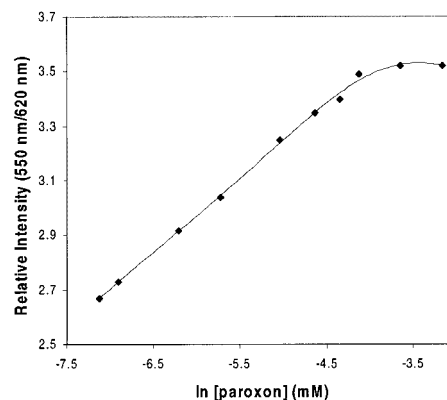


Figure 2. Fluorescence response of SNAFL-labeled organophosphorus hydrolase (approximately 30 $\mu\text{g OPH/cm}^3$ of PEG) chemically immobilized into PEG-1000 hydrogel spheres in 1 mM PBS (150 mM NaCl, pH 8.8) upon exposure to paraoxon.

weakly buffered solutions.²² The methacrylated PEG (MW 1000) exhibited a much lower pH shift of approximately 1.5 units. Therefore, while OPH reactivity with paraoxon is optimized between pH 8–9,²⁰ it was necessary to use more basic buffers in the solution surrounding the microspheres to provide the enzyme with an optimum microenvironment pH. The polymer-encapsulated fluorophore conjugate exhibited an emission redshift similar to that mentioned earlier.

Sphere fluorescent response was evaluated by titrating trace amounts of paraoxon into a cuvette loaded with spheres and 1 mM buffered PBS and then monitoring the change in the fluorescence emission ratio at 550 and 620 nm resulting from excitation at 510 nm. The acid peak fluorescent intensity increased with paraoxon concentration while spectra continued to pass through an isoemissive point at 620 nm, resulting in increasing fluorescent intensity ratios with higher concentrations of paraoxon. Spheres which were initially surrounded by a buffered solution higher in pH than the immobilized SNAFL pK_a experienced a visible color change from reddish purple to white as the microenvironment pH became acidic. An example response of PEG hydrogels (made from MW 1000 PEG dimethacrylate) to increasing paraoxon concentrations titrated into a 1 mM PBS buffer (pH 8.8) is presented in Figure 2. At low concentrations, the fluorescent response was linear with increasing paraoxon concentration. Unlike the solution results, a sensitivity plateau was reached at 15 μM of paraoxon. Response times for increases in paraoxon concentrations to as much as 10 μM were less than one minute (i.e., more rapid than the spectra acquisition time), while larger concentrations of paraoxon required as much as 10 min to reach a steady-state fluorescence ratio.

Examination of the cuvette revealed that both the fluorescent plateau and increasing response time at high paraoxon concentrations were likely the result of slow mass transfer of paraoxon and enzymatic degradation products between the hydrogel and solution. Experiments with large concentrations of paraoxon (and the resulting large changes in relative fluorescent ratio between 550/620 nm) conducted with intermediate mixing of the spheres resulted in a fluorescent response which continuously increased logarithmically with paraoxon concentration similar to electro-

(20) Rainina, E. I.; Efremenco, E. N.; Varfolomeyev, S. D.; Simonian, A. L.; Wild, J. R. *Biosens. Bioelectron.* **1996**, *11*, 991–1000.

(21) Agi, Y.; Walt, D. R. *J. Polym. Sci. Part A: Polym. Chem.* **1997**, *35*, 2105–2110.

(22) Silver, M. S.; Haskell, J. H. *Biochim. Biophys. Acta* **1990**, *1039*, 25–32.

chemical sensing results reported in the literature.²⁰ The relative fluorescent intensity (RFI) increase upon paraoxon addition had a slope of 0.046 per micromole of paraoxon in a concentration range between 3 and 100 μM .

The sphere fluorescence was regenerated from changes induced by paraoxon in less than 10 min by stirring in fresh PBS for lower concentrations. Higher concentrations of paraoxon generated more *p*-nitrophenol, which required additional time to regenerate the spheres. Hydrogel-immobilized enzymes stored at room temperature in normal room light were repeatedly used for more than one week without significant loss of activity and fluorescence intensity. Addition of paraoxon to the storage medium did not generate a fluorescence change or the production of *p*-nitrophenol, suggesting little or no leaching of enzyme from the hydrogels.

A recently reported amperometric OPH biosensor experimentally demonstrated discrimination of 2.3 μM paraoxon concentrations in water and suggested that on the basis of the sensor signal-to-noise characteristics the detection limit may be as low as 90 nM (for $S/N = 3$).¹² The lowest paraoxon concentration analyzed experimentally with our SNAFL-OPH sensor was 0.8 μM . Use of a self-referencing fluorescent dye reduces potential uncertainty from photobleaching and changes in sample geometry and results in a subsequent increase in S/N . A detection limit of 16 nM ($S/N = 3$) was estimated for the SNAFL-OPH biosensor.

CONCLUSIONS

Here we have demonstrated construction of a poly(ethylene glycol)-based optical sensor for neurotoxins using a self-referenc-

ing dye that has high sensitivity and stability under ambient conditions. This sensor configuration demonstrated over an order of magnitude sensitivity improvement as compared with other optically based OPH biosensors^{10,11} and equivalent sensitivity to that of the most recently reported amperometric OPH-based biosensors.¹² The combination of high substrate sensitivity, the nonfouling and enzyme stabilizing effects of PEG, and the ability to tailor OPH substrate selectivity may all be used to develop a sensitive, reusable neurotoxin biosensor for liquids and vapors. A current trend in artificial-nose development for odor recognition is "distributed specificity", in which a collection of different sensors each uniquely respond to a given stimulus.²³ An array of hydrogel beads containing several variants of mutant OPH enzymes could be optically interrogated to both detect and classify different neurotoxins based upon the substrate specificity of each individual recombinant enzyme.

ACKNOWLEDGMENT

M.V.P. and R.J.R. are grateful for support from the Texaco Corporation and the Whitaker Foundation (no. 98-581). A.L.S. and J.R.W. acknowledge contributions from the Department of Energy (Sandia National Laboratory at Livermore, no. LG3101) and the Texas Agriculture Experiment Station.

Received for review August 9, 1999. Accepted August 17, 1999.

AC990901U

(23) Dickinson, T.; White, J.; Kauer, J. S.; Walt, D. R. *Trends in Biotechnol.* **1998**, *16*, 250-258.