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Detection of Organophosphorus Compounds by Covalently Immobilized Organophosphorus Hydrolase

Jhony Orbulescu,[†] Celeste A. Constantine,[†] Vipin K. Rastogi,[‡] Saumil S. Shah,[‡] Joseph J. DeFrank,[‡] and Roger M. Leblanc*,[†]

Department of Chemistry, University of Miami, Coral Gables, Florida, 33146, and U.S. Army Edgewood Chemical Biological Center, Biotechnology Team, Research and Technology Directorate, Aberdeen Proving Ground, Maryland 21010-5423

As a consequence of organophosphorus (OP) toxins posing a threat to human life globally, organophosphorus hydrolase (OPH) has become the enzyme of choice to detoxify such compounds. Organophosphorus hydrolase was covalently immobilized onto a quartz substrate for utilization in paraoxon detection. The substrate was cleaned and modified prior to chemical attachment. Each modification step was monitored by imaging ellipsometry as the thickness increased with each modification step. The chemically attached OPH was labeled with a fluorescent dye (7-isothiocyanato-4-methylcoumarin) for the detection of paraoxon in aqueous solution, ranging from 10^{-9} to 10^{-5} M. UV−visible spectra were also acquired for the determination of the hydrolysis product of paraoxon, namely *p*-nitrophenol.

Organophosphorus (OP) derivatives are harmful compounds found in insecticides and pesticides and are widely utilized around the world.[1] These OP compounds are structurally similar to nerve gases and are well-known inhibitors of acetylcholinesterase, which is responsible for transmitting nerve impulses across synaptic junctions.[2] As a consequence of the acute toxicity of these neurotoxins, stringent environmental monitoring programs have been implemented to ensure that both groundwater and soil concentrations of OP derivatives remain below harmful toxic levels.[3] Paraoxon is the oxidative desulfuration product of the pesticide parathion when in contact with oxygen.[4] OP compounds are poisonous when ingested, inhaled, or absorbed through the skin. Therefore, development of sensors capable of determining harmful concentrations of theses compounds would be beneficial to both human and livestock populations.[3]

Organophosphorus hydrolase (OPH) enzyme can hydrolyze a large variety of organophosphorus compounds by producing less toxic products such as *p*-nitrophenol and diethyl phosphate.[5] OPH is one of the most studied enzymes related to its activity toward pesticides and nerve agents. It has been shown that OPH catalyzes hydrolysis reactions that induce cleavage in P−O, P−S, P−F, and P−CN bonds within organophosphorus neurotoxins.[6] Numerous analytical methods have been devised to detect the presence of OP compounds, e.g., gas chromatography,[7,8] high-performance liquid chromatography,[9,10] and other methods of detection, such as electrochemical.[11−14] Most of these techniques are time-consuming, expensive, and complex. To overcome these disadvantages, enzymatic bioassays have been designed for enhanced speed of detection, high efficiency, sensitivity, and cost-effectiveness.[15]

Several viable methodologies of enzyme immobilization are now explored.[16] Immobilization is important because it stabilizes the enzyme, and in many cases, the enzyme can be recovered and its activity regenerated.[17] These include physical immobilization,[18−20] encapsulation,[21−23] cross-linking,[24,25] and chemical bind-

* Corresponding author. Tel: +1-305-284-2194. Fax: +1-305-284-6367. E-mail: rml@miami.edu.

[†] University of Miami.

[‡] U.S. Army Edgewood Chemical Biological Center.

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ing to a solid substrate.[26−30] Covalent immobilization results in an increased stability since the attachment is not reversed by factors such as pH, ionic strength, substrate, solvent, or temperature.[31−33] The orientation can be predicted if small molecules or receptors are attached.[34] Metal oxide surfaces contain surface hydroxyl groups that are useful for the coupling of organic materials.[35] Carbon electrodes, after chemical treatment, possess different surface-associated functional groups such as carboxylic, carbonyl, lactone, and hydroxyl groups, which make the covalent attachment of enzymes possible.[36,37]

The number of ways an enzyme can be immobilized onto the solid surface is restricted by the groups that can be modified and present on the exterior of the enzyme "shell". Based on this restriction, we choose the modification of the free amine groups from any peripheral amino acids of the enzyme as is well known that amines can be modified easily without requiring harsh conditions that might affect the enzyme activity. In this investigation, quartz slides are functionalized with (3-aminopropyl)trimethoxysilane. The terminal amine group is then modified with diethylthiocarbamoyl chloride to form the isothiocyanate group. Isothiocyanates are known to react with amine groups in very mild conditions. OPH through the amine groups of the lysine, glutamic, or aspartic residues on the periphery of the enzyme[19,38,39] can react easily with the isothiocyanate groups from the substrate surface. Because the reaction occurs on the "outside" of the enzyme molecules, the binding sites of the OPH molecules remain available to interact with an external substrate.[40]

An enzyme can be utilized as a bioassay if it can provide qualitative and quantitative data regarding the reaction of interest, hydrolysis of OP compounds in this case. The hydrolysis product of paraoxon is *p*-nitrophenol, which has absorbance in UV region, but even with its relatively high extinction coefficient ($\epsilon_{400} = 18\,000$ $M^{-1}\cdot cm^{-1}$), only fairly concentrated solutions of *p*-nitrophenol can be measured by UV−visible spectroscopy. It is well known that fluorescence spectroscopy can detect much lower concentrations than UV−visible spectroscopy. Based on this fact, we decided to label the OPH enzyme with a reliable fluorophore whose emission can be affected by the formation in solution of the *p*-nitrophenol during paraoxon hydrolysis. Coumarin is a widely used fluorophore; this UV-excitable dye possesses excellent spectroscopic properties, e.g., large Stokes shift (larger than 80 nm) and medium quantum efficiency of fluorescence ($\Phi = 0.5$). These key characteristics make coumarin derivatives very attractive for use as labels.[41,42] In addition, the emission of the coumarin dye (~430 nm) can overlap with the absorbance of the hydrolysis product, namely, *p*-nitrophenol (~400 nm), leading to fluorescence resonance energy transfer (FRET) that can quench the coumarin emission.

In the present investigation, covalent binding of OPH to a silanized quartz slide was performed. Diethylthiocarbamoyl chloride was employed as a reagent for modifying the amino-modified quartz slide surface, which was then used for covalently attaching enzymes. OPH was then labeled with a coumarin derivative fluorophore. This sensing device was utilized in monitoring the paraoxon hydrolysis reaction using UV−visible and fluorescence spectroscopies.

EXPERIMENTAL SECTION

The quartz slides were obtained from Hellma Inc. (Plainview, NY). (3-Aminopropyl)trimethoxysilane used for the silanization of the quartz slide and diethylthiocarbamoyl chloride were obtained from Aldrich (Milwauke, WI). Carbonate−bicarbonate buffer capsules, pH 9.6, were purchased from Sigma (St. Louis, MO). Spectroscopic grade solvents were obtained from Fischer Scientific (Fair Lawn, NJ). The 7-isothiocyanato-4-methylcoumarin was synthesized from the commercially available 7-amino-4-methylcoumarin obtained from Aldrich (Milwaukee, WI).[43] OPH (85−90%) (EC 3.1.8.1) was isolated, extracted, and purified at the U.S. Army Laboratory (Edgewood Chemical and Biological Center, MD). A stock solution of OPH (1.8 mg/mL) was prepared in 100 mM bis-tris-propane, pH 7.3, containing 10 μM Co^{2+}. The stock solution was frozen at −4 °C. The stock solution was diluted to a concentration of 0.18 mg/mL prior to use. Pure water was provided from the Modulab 2020 water purification system (Continental Water Systems Corp., San Antonio, TX). The resistance and surface tension of pure water were 18 MΩ·cm and 72.6 mN/m at 20.0 ± 0.5 °C, respectively. Attenuated total reflectance (ATR)-FT-IR spectra were recorded on an Equinox 55 FT-IR spectrometer (Bruker Optics Inc., Billerica, MA) with a 25 reflection variable angle ATR P/N1 1000 (Specac Inc., Smyrna, GA) accessory. The modulation frequency was set at 1666 cm^{-1}, and 300 scans were collected for each spectrum at a resolution of 4 cm^{-1}. KRS-5 crystal was used, and the incidence angle was set at 60°. The changes in thickness of the films upon modification were measured using an I-Elli2000 imaging ellipsometer (Nanofilm, Göttingen, Germany). A Lambda 900 UV−visible−NIR spectrophotometer (Perkin-Elmer, Boston, MA) using a quartz cuvette of 1-cm optical path length was used to measure the absorption spectra. The fluorescence spectra were obtained using a Spex Fluorolog 1680 spectrophotometer (Jobin Yvon, Inc., Edison, NJ).

Covalent Immobilization of OPH−Coumarin. Sample substrates were cleaned by immersion in a chromic mixture and

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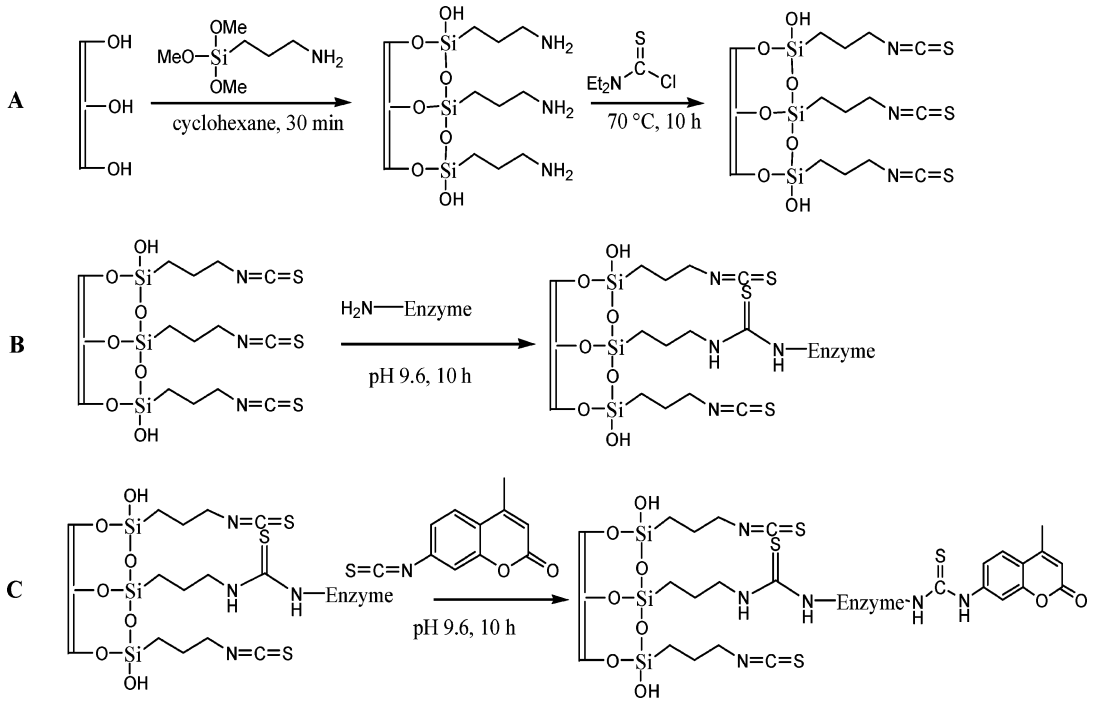
Scheme 1. Reaction Steps for OPH Immobilization: (A) Surface Silanization and Activation; (B) Enzyme Immobilization, (C) Immobilized Enzyme Labeling



sonicated for 30 min. This was followed by alternate rinsing and sonication. The steps showing the covalent binding of OPH are presented in Scheme 1. In the first step prior to immobilization of OPH, the substrate cleaned and activated (OH groups on the surface), was silanized by reaction with (3-aminopropyl)trimethoxysilane.[44] The amino-modified slides were reacted with diethylthiocarbamoyl chloride at 70 °C for 10 h in order to convert the amino to isothiocyanate groups. The isothiocyanate functional group was then used for covalent binding of OPH.[45] This process required another 10-h incubation time while the functionalized quartz slides were immersed in an aqueous buffered solution of OPH (0.2 mg/mL OPH in 0.05 M carbonate−bicarbonate buffer, pH 9.6). After immobilization, the slides were washed thoroughly to remove all unreacted and physically adsorbed OPH. The covalently attached OPH was labeled with 7-isothiocyanato-4-methylcoumarin for fluorescence spectroscopic studies. The covalently immobilized OPH−coumarin quartz slides were stored in a phosphate buffer solution (pH 8.6) prior to use.

RESULTS AND DISCUSSION

Characterization of the covalent reaction. ATR-IR spectroscopy was utilized to confirm the functionality of the new bonds formed. Two bands were noted at 2853 and 2924 cm^{-1} after the first step of the reaction (Scheme 1A), and these peaks represent the C−H symmetric and asymmetric stretching modes, respectively, which is due to the presence of the alkyl chain (Figure 1). Although the amino group could not be observed from the ATR spectrum, the C−H vibration provides strong enough evidence to confirm the initial silanization step. Hereafter, the quartz slide was essentially prepared for the following steps where the amino groups were reacted with diethylthiocarbamoyl chloride producing the isothiocyanatopropyl-modified slide (Scheme 1A, step 2). The isothiocyanate groups subsequently reacted with the amino groups on the surface of OPH. The secondary structure of OPH was investigated to confirm the presence of the enzyme (Figure 2). The IR spectrum of the enzyme shows the amide I and II bands, which are in the region of 1700−1600 and 1600−1500 cm^{-1}, respectively. The two amide regions were clearly observed. The amide I region contains the greatest information for the analysis of enzyme secondary structure. The peaks were assigned to the corresponding secondary structure as shown in Table 1. It can be seen that the covalently bound OPH film exhibited absorbance bands for both the α-helix and β-sheet conformations. The frequency peaks at 1657, 1647, 1550, and 1534 cm^{-1} correspond to the α-helix and those at 1693, 1679, 1634, and 1619 cm^{-1} correspond to the β-sheet conformations.

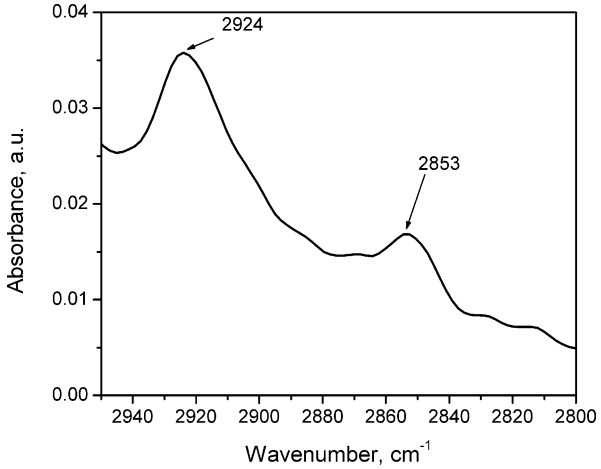


Figure 1. ATR-IR spectrum of silanized quartz slide.

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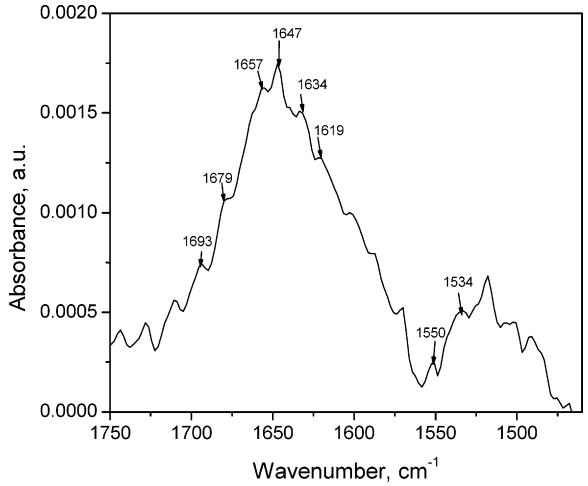

Figure 2. ATR-IR spectrum of covalently bound OPH.

Table 1. IR Band Assignment Indicating the Secondary Structure of OPH Covalently Bound to the Quartz Slide

frequency band position (cm^{-1})	assignment
1693	antiparallel β-sheet or pleated turn
1679	β-sheet
1657	α-helix
1647	amide I
1634	β-sheet
1619	β-sheet
1550	α-helix
1534	amide II

Imaging ellipsometry was also used to characterize each step of the reaction scheme (see Scheme 1). The thickness for each step was calculated directly from the ellipsometric angles where the incident angle was set up at 53°. To calculate the thickness (d) for each step, an optical model was used that consisted of several parameters. The refractive index (n) and extinction coefficient (k) were 1.46 and 0, respectively, for the substrate used. The refractive index for air and for the film was 1.0 and 1.50, respectively. The amplitude (Δ) and the phase (ψ) of the wave was 171.13 and 4.97, respectively. The wavelength of the laser was at 532 nm. The average thickness generated in the 2-D ellipsometric images in Figure 3A and B are indicated by the red color. Figure 3A presents the thickness map generated in both 2-D and 3-D for the silanization step (Scheme 1A, step 1). The average thickness of the silanization step was determined to be ~10 Å. This was in close agreement with the CPK value of 8 Å. The scale of −5 to +5 nm, on the right side of the image, has no physical meaning and it was used only for a better illustration in terms of colors for the measured thickness. The 3-D map (Figure 3B) shows a homogeneous surface. The second step (Scheme 1A, step 2) was not imaged because the resolution of the instrument is ±1 Å and a theoretical value of 9 Å is expected, which is ~1 Å thicker than the first step. The third step (Scheme 1B), which indicates the covalent attachment of OPH, is shown in Figure 3B. The average thickness generated is between 50 and 60 Å. An expected value of 58 Å was calculated based on the X-ray crystalline structure of OPH with an overall dimension of approximately 51 Å × 55 Å × 51 Å.[46] There is a good correlation between the experimental average value and the theoretical one. It can be seen that the surface is not covered homogeneously with the OPH enzyme. This is very likely the effect of the mild experimental conditions used in the OPH attachment in order to maintain the enzyme activity unaltered. As a last step, the chemically attached OPH was labeled with 7-isothiocyanato-4-methylcoumarin by reacting free amine groups from the enzyme with the isothiocyanate from the coumarin (Scheme 1C). Following surface modification and enzyme labeling, the modified slides called thereafter a sensing device was used for the investigation of the paraoxon hydrolysis by the surface-attached OPH using spectroscopic methods.

UV−Visible Absorption Studies of Covalently Immobilized OPH. To ensure that the covalently attached OPH was labeled with the coumarin derivative, a UV−visible absorption spectrum of the sensing device was measured and compared to the one of 7-isothiocyanato-4-methylcoumarin in aqueous solution. Figure 4 shows spectrum 1 of the coumarin derivative in aqueous solution with an absorption band situated at 341 nm characteristic of the coumarin derivatives. The presence of the isothiocyanate group at position 7 shifts the absorption maximum to shorter wavelength as shown by spectrum 2.[47] This spectrum confirms the presence of the coumarin attached to the OPH enzyme.

The covalently attached OPH−coumarin was exposed to paraoxon solution, which was hydrolyzed by OPH into its products diethyl phosphate and *p*-nitrophenol. The UV−visible absorption spectrum of paraoxon solution (1×10^{-6} M) is shown in Figure 5, curve 1 with an absorption maximum at 274 nm. After recording the absorption spectrum of the paraoxon solution in a 1-cm optical path length cuvette, the sensing device was placed into the solution for 30 s. The sensing device was then removed and the absorption spectrum of the solution measured again. A new band situated at 400 nm was observed and corresponds to the absorption maximum of the reaction product, namely, *p*-nitrophenol (Figure 5, curve 2). The process of placing and removing the sensing device from the paraoxon solution at different time periods permitted us to follow the kinetics of *p*-nitrophenol formation. As shown in the inset in Figure 5, the decrease in paraoxon absorption at 274 nm was paralleled by an increase in *p*-nitrophenol absorption at 400 nm as exposure time of the sensing device increased. The symmetry (inset in Figure 5) between the decrease in absorbance for the paraoxon and the increase in absorbance for the generated *p*-nitrophenol is a direct proof that an amount of paraoxon is hydrolyzed by the OPH enzyme and a similar amount of *p*-nitrophenol is formed, and no other effects are involved in the changes in the absorbance.

An isosbestic point at 318 nm in the absorption spectrum as shown in Figure 5 was observed, and this suggests the presence of two species, namely, paraoxon as reactant and *p*-nitrophenol as product; no other intermediates were involved in the hydrolysis reaction. The meaning of the isosbestic point is that the total concentration of the two species is a constant and when the concentration of paraoxon is decreasing a similar increase in the *p*-nitrophenol concentration is observed. In addition, the molar absorptivity of the two species is constant at 318 nm. The sensing device's fast detection time was shown by the ability to detect

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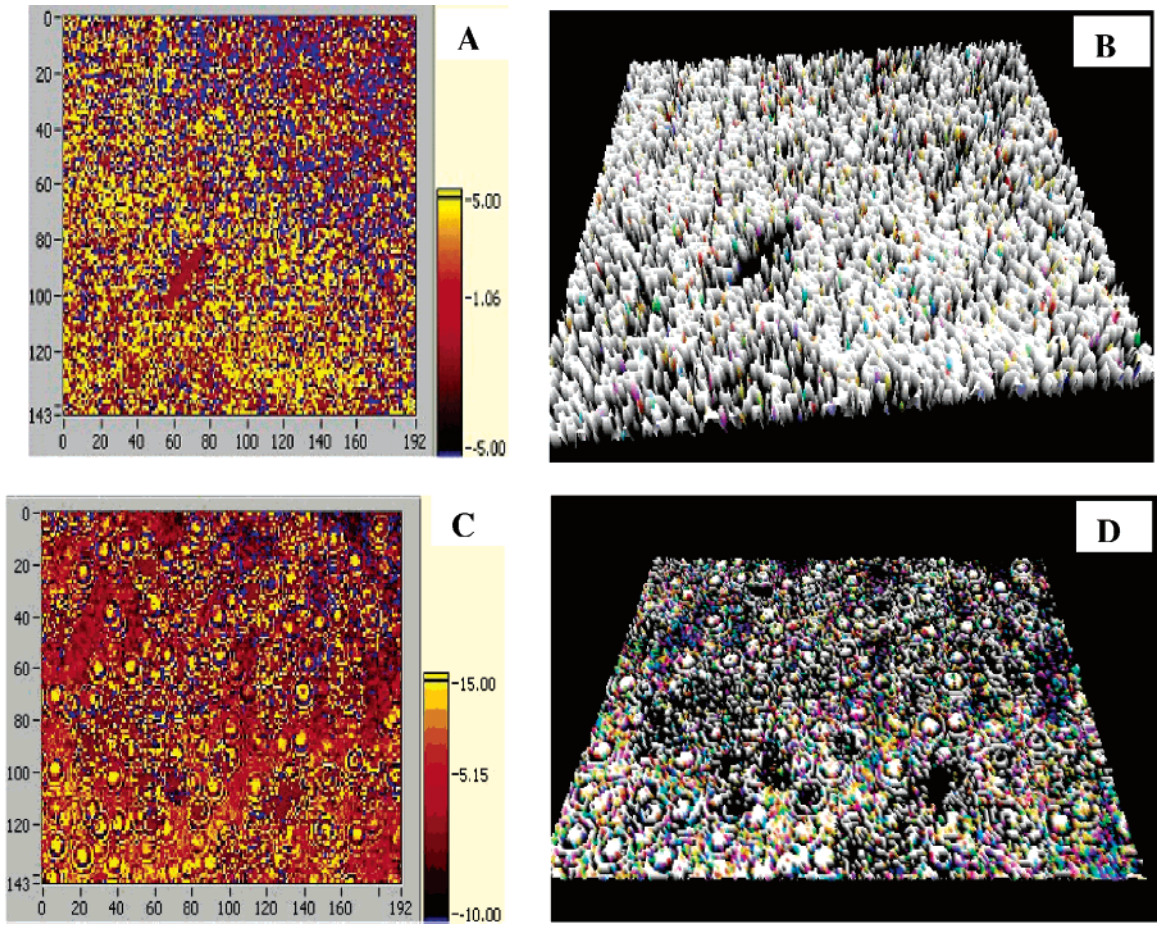

Figure 3. Ellipsometric characterization: (A) and (B) 2-D and 3-D images, respectively, of silanized quartz slide; (C) and (D) 2-D and 3-D images, respectively, of covalently bound OPH.

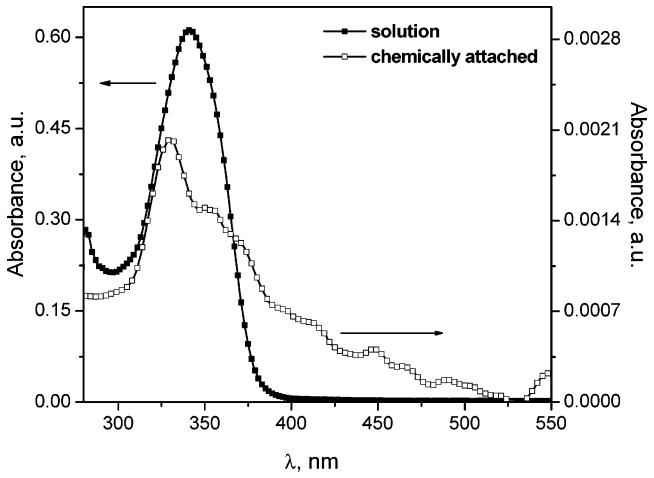

Figure 4. UV−visible spectra of 7-isothiocyanato-4-methylcoumarin in solution (1×10^{-6} M, 1-cm optical path length) (■) and as a label on immobilized OPH (□).

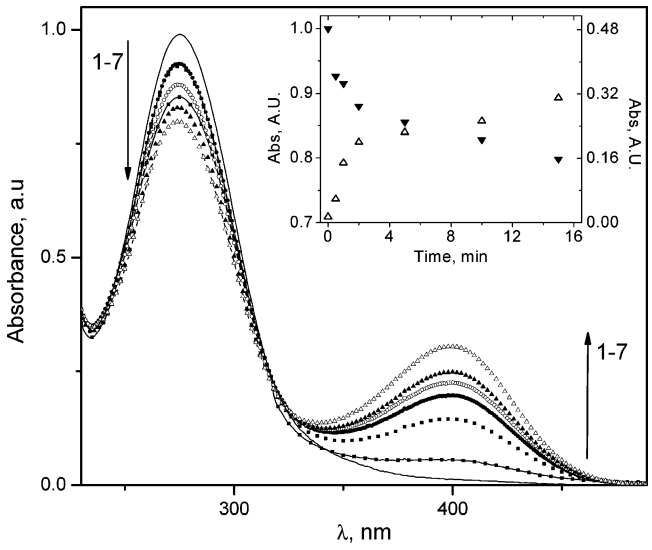

Figure 5. UV−visible absorption spectra of the hydrolysis product *p*-nitrophenol at different time intervals for the detection of paraoxon solution (1) 1×10^{-6} M. Incubation time of the sensing device (see last modification step in Scheme 1) in paraoxon solution: (2) 30 s and (3−7) 1, 2, 5, 10, and 15 min. Inset: decrease in paraoxon absorption (▼) and the corresponding increase in *p*-nitrophenol absorption (△); 1-cm optical path length.

p-nitrophenol after only 30 s of exposure. We interpret the waiting time of 30 s as time needed for the accumulation of sufficient *p*-nitrophenol in solution for detection. Our reasoning is based on the previous work of Benning et al.[48] and Omburo et al.,[49] who showed that the paraoxon hydrolysis occurs very fast, as suggested by their kinetic measurements, i.e., $K_m = 45 \pm 5$, $k_{cat} = 1520 \pm 40$, and $k_{cat}/K_m = 3.4 \pm 0.3$. The mechanism and kinetics

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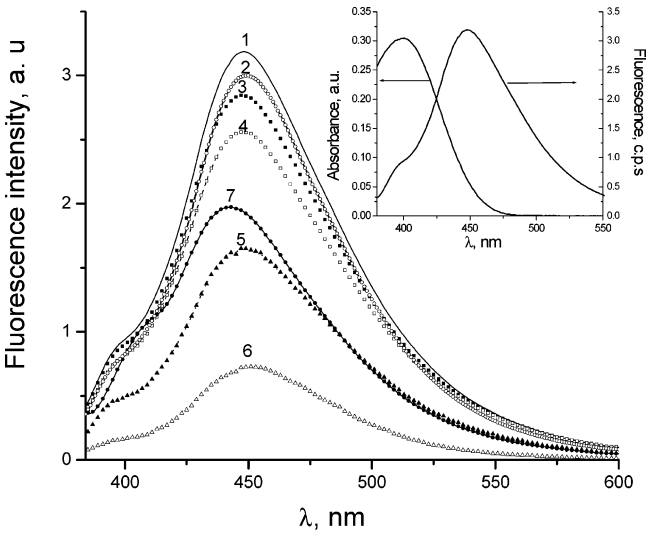

Figure 6. Fluorescence spectra of coumarin-labeled immobilized OPH in the presence of different concentrations of paraoxon solution (λ_{exc} = 340 nm, 1-cm optical path length): (1) solid substrate labeled with OPH−coumarin, (2) 5×10^{-9}, (3) 5×10^{-8}, (4) 5×10^{-7}, (5) 1×10^{-6}, and (6) 1×10^{-5} M, and (7) recovery of slide. Inset: overlap between the absorption of the acceptor (*p*-nitrophenol) and the fluorescence of the donor (coumarin-labeled enzyme).

of paraoxon hydrolysis were recently studied in detail by Aubert et al.,[50] who showed that among four substrates investigated the kinetics appeared faster for paraoxon. It was also reported by Briseño-Roa et al.[51] that, among the organophosphorus compounds studied, the hydrolysis reaction was faster for paraoxon. Since the catalysis step is fast, the 30 s was needed to accumulate *p*-nitrophenol at a concentration measurable by UV−visible absorption spectroscopy. This interpretation is in agreement with the data reported by Caldwell et al.[52] about the effect of solvent viscosity and structure−reactivity relationships.

Fluorescence Emission Spectroscopic Studies of Covalently Immobilized OPH. Fluorescence measurements were recorded at different concentrations of paraoxon solution when exposed to the sensing device. From the UV−visible spectroscopic data, it was determined that the analyte required 30 s of exposure to the sensing device in order to detect the hydrolysis product, *p*-nitrophenol. Increasing concentration of paraoxon solutions were exposed to the sensing device, and the results are presented in Figure 6. The initial fluorescence intensity of the fluorophore is shown by curve 1 (λ_{Ex} = 350 nm, λ_{Em} = 445 nm). On exposure to paraoxon solution, there is a slight decrease of the fluorescence intensity (curves 1−4) followed by a more drastic quenching of the fluorescence intensity (curves 5 and 6) as the chemically bound enzyme hydrolyses paraoxon into *p*-nitrophenol. The mechanism responsible for the quenching of the fluorescence intensity is attributed to the Förster FRET. The inset of Figure 6 presents the spectral overlap between the absorption of *p*-nitrophenol (acceptor) and fluorescence of the coumarin fluorophore (donor). As paraoxon is hydrolyzed, excited-state energy from the initially excited 7-isothiocyanato-4-methylcoumarin is transferred to *p*-nitrophenol, thereby leading to the observed quenching phenomenon. After enzyme-bound quartz slides were used, they were washed in water and KH_2PO_4−NaOH buffer solution (pH 8.6). Curve 7 shows the fluorescence intensity after recovery. This process removed some of the product from the sensing device. Removal of the product allowed the coumarin label to regain 60% of its fluorescence intensity. Possible reasons for only 60% recovery of the fluorescence are related to the fact that the enzyme-bound quartz slide used for coumarin labeling was not sonicated but rinsed with solvent, and secondary structure alteration can occur if sonication is used. It is possible that some physically adsorbed enzyme was present on the surface. Following the experiments, storage of the modified slide in buffer solution could remove the adsorbed enzyme; thus, the recovery would give a lower percent that the initial emission of the labeled attached enzyme measured before the experiments. Second and most probable is the presence of some coumarin derivative that interacted with the enzyme surface but not chemically bonded. If so, storing the slide in buffer solution could remove the coumarin molecules adsorbed onto the enzyme surface by hydrogen bonding or $\pi-\pi$ stacking between the aromatic ring of the coumarin and aromatic side chains of tyrosine, tryptophan, or phenylalanine present on the enzyme surface.

CONCLUSION

Silanization was successfully performed to functionalize a quartz substrate in order to provide an environment suitable for the covalent binding of OPH. Rapid detection of paraoxon can be carried out using both UV−visible and fluorescence spectroscopies. Fluorescence spectroscopy is more sensitive and allows a lower detection limit (5×10^{-9} M) than UV−visible spectroscopy. The results were reproducible and show that the fluorescence intensity of the immobilized OPH−coumarin was partially recovered (60%). Based on the sensitivity and high efficiency of the sensing device, it could lead to a prototype for an industrial biosensor that can be implemented for environmental monitoring of organophosphorus compounds.

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