Optical leaky waveguide biosensors for the detection of organophosphorus pesticides

M. Zourob,†*[a] A. Simonian,[b] J. Wild,[c] S. Mohr,[a] Xudong Fan,[d] I. Abdulhalim[e] and N. J. Goddard[a]

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Organophosphorus (OP) pesticides can be rapidly detected by integrating organophosphorus hydrolase with an optical leaky waveguide biosensor. This enzyme catalyses the hydrolysis of a wide range of organophosphorus compounds causing an increase in the pH. Thus, the direct detection of OP is possible by monitoring of the pH changes associated with the enzyme's activity. This article describes the use of an optical, leaky waveguide clad with absorbing materials for the detection of OP pesticides by measuring changes in refractive index, absorbance and fluorescence. In the most effective configuration, a thick sensing layer was used to increase the amount of immobilized enzyme and to increase the light interaction with the sensing layer, resulting in a greatly enhanced sensitivity. The platforms developed in this work were successfully used to detect paraoxon and parathion down to 4 nM concentrations.

1. Introduction

Neurotoxic organophosphorus (OP) compounds are commonly used as pesticides and chemical warfare agents (CWA). These neurotoxic compounds irreversibly inhibit the enzyme acetylcholinesterase, resulting in the build up of the neurotransmitter acetylcholine, which interferes with the muscarinic responses in vital organs, producing serious symptoms and eventually leading to death.[1,2] Early detection of OP neurotoxins is important in the effective defence against terrorist activity, for protection of water resources, and for monitoring the safety of food supplies, as well as for monitoring the progress of detoxification processes.

Different techniques have been used for the detection of organophosphorus pesticides, such as solid-phase microextraction–gas chromatography procedures,[3–5] gas chromatography–mass spectrometry,[6] liquid chromatography,[7] capillary electrophoresis,[8,9] and ion mobility spectrometry.[10] Although these techniques offer high sensitivity and exquisite selectivity, they are not suitable for use as rapid screening methods nor can they be used for field applications because of their technical sophistication (lack of "hardness") and the intrusion of environmental contaminations.[11] Selective, hardened biosensors appear to be a promising technology to overcome these problems. Therefore, an intense interest has emerged for the development of biosensors for highly sensitive, selective and rapid on-site determination of OP. Several types of biosensors based on different signal transduction mechanisms have been reported for the detection of OP including amperometric,[12–18] potentiometric[11,19–25] and optical techniques.[26–33] These devices used enzyme sensors based on acetylcholinesterase inhibition,[14,29,31,34–36] organophosphorus hydrolase,[21,23,24,28,35,37–39] organophosphorus acid anhydrolase,[40] genetically engineered cells[11,19,41–46] and immunosensors based on anti-pesticide antibodies.[47,48]

The enzymatic techniques relying on the inhibition of acetylcholinesterase (AChE) are not selective (due to their conflicting responses to a wide range of toxic inhibitors),[35] and the assays are indirect and slow due to the requirements of prolonged incubation and regeneration periods. In addition, due to the irreversible nature of enzyme–ligand interactions, inhibition-mode sensors can not be reused without regeneration of enzyme activity. In many cases this is inefficient and time-consuming:[24] however, the use of organophosphoros hydrolase (OPH) as the enzyme transducer can overcome these concerns as it is fast (k_{cat} = 300 to 8000 s^{-1} depending on the OP), more selective for OP compounds, and subject to direct detection.[11,35,37] Organophosphorus hydrolase (OPH, E.C. 3.1.8.1) is an enzyme which can hydrolyze phosphotriesters, phosphonothioates, phosphonofluoridates, and phosphonocyanidates. This unique enzyme catalyzes the hydrolysis of a wide range of OP, and such activity has been exploited in biosensors.[11,13,20,23,35,37,49–53]

Optical biosensors have been the subject of intense interest over the past two decades due to their numerous advantages. They can be miniaturised, they have multiplexing capabilities, and the optical biosensor technology combines rapid response times with high sensitivity for analyte evaluation. Hence, their use in real-time detection of OP appears promising.

This article describes the use of absorbing materials-clad leaky waveguide biosensors for the detection of OP pesticides. The present work investigates the possibility of producing low cost polymer chips with sol-gel and hydrogel as guiding layers

[a]*School of Chemical Engineering and Analytical Science, University of Manchester, Manchester, UK. E-mail: m.zourob@manchester.ac.uk; Fax: +44 161 200 4911; Tel: +44 161 200 8884*
[b]*Mechanical Engineering Department, Auburn University, Auburn, AL, USA*
[c]*Biochemistry & Biophysics Department, Texas A&M University, College Station, TX, USA*
[d]*Department of Biological Engineering, University of Missouri-Columbia, Columbia, MO, USA*
[e]*Department of Electro-Optics Engineering, Ben Gurion University, Israel*
† Current address: Institute of Biotechnology, University of Cambridge, Cambridge, UK.

as well as for the entrapment of enzymes. The biosensor platforms developed in this work were successfully used to detect OP by using organophosphorus hydrolase, immobilised within a sol-gel/hydrogel matrix, by monitoring the changes in refractive index, absorbance and fluorescence.

2. Absorbing materials-clad leaky waveguide

Currently, the main focus in developing chemical sensors for routine use is to produce systems which have sufficient sensing performance, use fewer materials, have lower fabrication time, produce lower manufacturing costs, and be as user friendly as possible. The use of polymer materials together with micro-system technology offers the most promise of fulfilling these aims. Such effective materials are applicable for mass replication technologies, such as injection moulding and hot embossing, as well as for methods of prototyping.

Previous studies have evaluated the feasibility of using alternative, simpler methods for the production of low-cost evanescent, optical leaky waveguide devices for the detection of refractive index (RI) and fluorescence of bulk solutions in μ-total analysis systems (μ-TAS) systems and bacteria detection.[54,55] Different absorbing materials can be used, such as dyes or polymers rather than metals. These systems can use room temperature fabrication techniques to deposit the layers, which is an easier approach, more friendly for the biological materials, and considerably less expensive. These alternatives were found to produce no significant change in modulation or width of the resonances in the angular reflectivity.[54,55]

In this article the authors discuss the development of new formats of absorbing materials-clad optical leaky waveguides for chemical detection. In this format, the absorbing materials clad layer is sandwiched between the polymer substrate and the sensing layer. This format consists of a polymethylmethacrylate (PMMA) polymer substrate coated with an ~30 nm thick absorbing layer followed by a thick sensing layer where the enzyme is entrapped. When the hydrogel was used, a 3 μm thickness was chosen as sensing layer, and in case of the sol-gel platform as the sensing layer, a 2 μm thickness was used. A typical profile for the designed polythiophene clad-leaky waveguide (PT)-CLW is shown in Fig. 1.

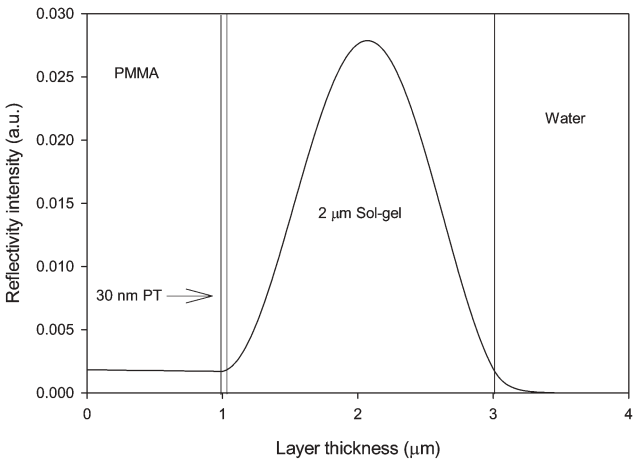

Fig. 1 Mode profile of PT-CLW at 470 nm.

The advantage of inserting the absorbing material layer is that it absorbs a large proportion of any scattered light or auto-fluorescent background from the polymer substrate. This is particularly useful when the injection moulded chips with the integrated grating are being used, hence optical anisotropy is introduced into the finished device. The thickness of the waveguiding layer of the devices were designed to support one, sharp-guided mode (TE_0) and to allow the maximum interaction of the light with the sensing layer, where the enzyme is entrapped along with the suitable indicator. This thick sensing layer increases the amount of entrapped enzyme within the sensing layer, and results in an enhanced sensitivity through increasing the change in the pH around the enzyme. This arrangement allowed the maximum interaction with the entrapped enzyme, resulting in a higher sensitivity.

Experimental

Materials

Paraoxon, parathion and diazinon were purchased from Supelco (Gillingham, Dorset, UK). Wild-type organophosphorus hydrolase (OPH) was isolated and purified from a recombinant *Escherichia coli* strain using published procedures.[49,50] Solvent blue, poly(3-hexylthiophene-2,5-diyl), chloroform, fluorescein isothiocyanate (FITC), Ches and phosphate buffer saline (PBS), acetonitrile, 2-hydroxyethyl methacrylate (HEMA), ethylene glycol dimethacrylate (EDMA), methacrylic acid (MAA), 2-dimethoxy-2-phenyl acetophenone (DMPA) and 3-(trimethoxysilyl)-propyl methacrylate were purchased from Aldrich (Gillingham, Dorset, UK). UV-curable optical adhesive Norland 61 was purchased from Norland Products Inc. (New Brunswick, NJ, USA). UV-curable aliphatic fluorinated polyacrylate-co-methacrylate (UV-Opti-Clad1.413XM) with refractive index n = 1.413 was purchased from Optical Polymer Research, Inc., (Gianesville, Florida, USA). A master relief diffraction grating (25 400 lines per inch) was purchased from Edmund Optics (York, UK).

Instrumentation

A schematic of the optical arrangement used is shown in Fig. 2. For the detection of refractive index changes the optical arrangement has been detailed elsewhere.[54,55] The LED used in the input side had a peak wavelength of 610 nm (RS components, Corby, UK). The LED was polished flat and mounted at the end of a 25 mm diameter mounting tube. The mounting tube incorporated a collimating lens with a 40 mm focal length and cylindrical lens with a 75 mm focal length in order to focus the light into a line. An interference filter (610 nm ± 10 nm peak wavelength), and a 25 mm in diameter was also used. All optical components were obtained from Comar Instruments (Cambridge, UK), unless otherwise stated. Fluorescence measurements were performed using the same optical set-up as described before except a blue LED, (peak wavelength 470 nm) as excitation light source and an interference filter (470 nm ± 10 nm) were used.

An epoxy replica of a holographically-patterned grating (25 400 lines per inch, Edmund Optics) was moulded to one side of the chip in order to couple the light into the waveguide.

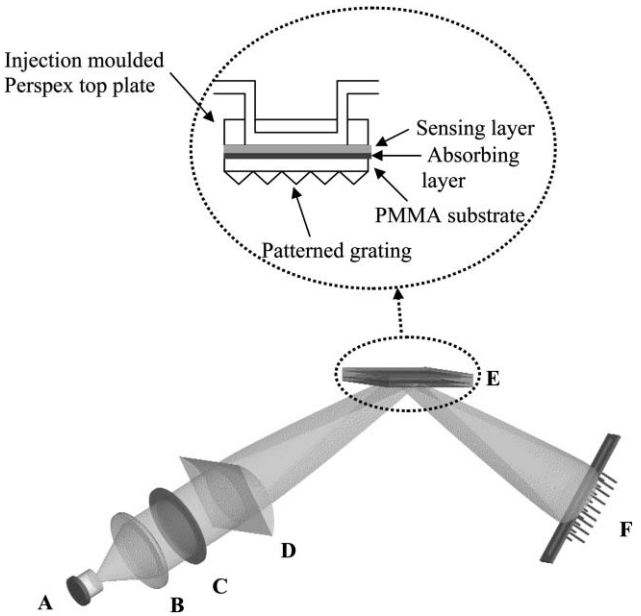

Fig. 2 Sensor optical design using (a) 473 nm LED or 610 nm LED, (b) collimating lens, (c) filter, (d) cylindrical lens, (e) sensor chip, (f) linear CCD detector.

On the output side, a 8000 pixel linear CCD (Sony ILX506A, pixel pitch 7 μm) was positioned at a distance of 12 cm, and was used to monitor the resonance angle and intensity changes in the guided modes. Data was collected by a 12-bit analogue-to-digital converter with a high-speed parallel link to a computer running with software written in-house. In the case of fluorescence measurements a band pass filter (510 nm ± 20 nm) (Glen Spectra, Stanmore, UK), and home-made collimating optics were placed in front of the CCD detector.

A peristaltic pump (MINIPULS-3, MP4, Gilson, Canada) was used to pump solutions at 50 μl min^{-1} through the flow cell.

Dye-clad leaky waveguide chip fabrication

Injection moulding of the PMMA substrates containing the grating coupler was performed as previously described.[56,57] A holographic grating film (25 400 lines per inch, Edmund Optics) was first treated to reduce the adhesion to the epoxy from which the grating was to be moulded by being soaked in saturated sodium dodecyl sulfate solution for 20 minutes, washed in copious water and dried under a stream of dry nitrogen.[56,57] A few drops of UV-curable epoxy (Norland 81) (Norland Products Inc., New Brunswick, NJ) were then placed onto the centre of a laminar polished surface of the glass block and the grating film was placed on top. Capillary action formed an even layer of epoxy over the entire glass surface before UV exposure (365 nm). The chip was rapidly cooled over cardice for 30 seconds to facilitate easy removal of the holographic film and then dried under a stream of dry nitrogen before a second, short UV exposure. The glass block was placed in the oven at 50 °C for 6 h before being used as a master for injection moulding. Injection moulded Poly-(dimethylmethacrylate) (PMMA) chips were produced using a Babyplast 6/6 (Cronoplast S. L., Barcelona, Spain) injection-moulding machine.

The absorbing material waveguide devices required three layers: a PMMA substrate, an absorbing material (solvent blue or polythiophene (PT) clad layer), and a waveguide sensing layer (hydrogel or sol-gel). The wavelength range of interest determined the materials used. Solvent blue 35 was selected for 610 nm wavelength, and a polymer, polythiophene (PT), was selected for 470 nm wavelength. The absorbing materials-CLW chips were fabricated by depositing the material onto the PMMA substrate. The PT solution was prepared by dissolving 15 mg polythiophene in 10 ml chloroform overnight. The solvent blue 35 solution was prepared by dissolving 50 mg solvent blue in 10 ml ethyl acetate containing 0.1 g of UV curable polymer (UV-Opti-Clad 1.413XM). The dye and PT solutions were spin-coated onto the PMMA substrate at 4000 rpm. The dye and UV curable polymer clad layers were exposed to the UV light for 1 min while the PT clad layers were left to dry overnight in the oven at 60 °C.

Sensor preparation

Gel film preparation

Poly-HEMA gel containing 5 mol% ethylene glycol dimethyacrylate (EDMA) and 6 mol% of the functional momoners methacrylic acid were prepared in equal volumes of isopropanol and water. Then 2% (w/v) DMPA was added and mixed for extra five minutes, and the solution was degassed in a stream of nitrogen prior to UV exposure. The HEMA was exposed to 30-second pulses of UV light (to grow the polymer), mixed, degassed, and exposed to another UV-light exposure. This process was repeated four times until the HEMA solution adopted a thick consistency. 1 ml of the HEMA solution was spin coated onto a silanized modified chip with methacryloxypropyltriethoxysilane as described previously[58] at 4000 rpm for 30 seconds. The chip was exposed to the UV light again for an extra 5 min for full curing.

The cured gel was rehydrated with phosphate buffer saline (PBS, pH 8.3, 20 mM phosphate, 100 mM NaCl, 2.7 mM KCl, 30 mM $CoCl_2$) for 30 minutes before enzyme immobilization as described previously.[59] 100 μL of OPH solution (1 mg ml^{-1} in PBS, pH 8.3, 10 mM, 2.7 mM KCl) was covering the entire waveguiding hydrogel layer in a fume-hood for 1 h. Covalent immobilization was achieved by addition of 100 μL of 1.25% glutaradehyde solution (diluted from 25% (v/v) glutaraldehyde solution) in phosphate buffer on to the waveguiding surface and leaving for 2 h. Control waveguiding layers were also constructed by immobilizing bovine serum albumin (1mg ml^{-1} in PBS, pH 8.3, 10 mM) using the same procedure.

Sol-gel preparation

To prepare the sol-gel waveguiding layer, the sol-gel precursors (2.15 g tetraethoxysilane (TEOS), 1.85 g of methyltriethoxysilane (MTEOS)) were mixed together. In the case of absorbance measurements, 20 mg bromocresol green (BCG) acid–base indicator was completely dissolved in the sol-gel precursors. Next 2.4 ml of 0.04 M HCl was added dropwise

and stirred using a magnetic stirrer for 1.5 h to form the sol-gel. Subsequently, 3 ml of the sol-gel and 3 ml of 10 mM phosphate buffer (pH 8.3) were cooled over ice for 20 min. The buffer was then added slowly to the sol-gel while stirring over ice. After 2 min, 100 μl of OPH was added and the solution mixed for a further two min. Finally, the mixture was spin coated on top of the chip at 3000 rpm.

Results and discussion

Refractive index detection

In the work presented here, the solvent blue-clad leaky waveguide chip was used for detecting OP pesticides by monitoring refractive index changes. This was achieved by immobilizing the OPH enzyme within a pH-sensitive hydrogel waveguiding layer. It is known that OPH catalyses the hydrolysis of a broad range of OP pesticide compounds, including the P–O, P–F, P–S, or P–CN bonds in the OP compounds. The reaction can generate up to two protons as a result of each hydrolysis reaction (Fig. 3), thus making it possible to direct neurotoxin detection by measurement of the pH change associated with the enzyme activity.[11] Using one or more ionic or ionisable monomers in a cross-linked polymeric backbone makes it possible to detect the change in the pH around the enzyme as a result of influencing the charge of the ionic monomers.[59,60] This causes the hydrogel to swell or to shrink depending on the degree of influence on the charges of the ionic monomers, which can be detected easily by the optical waveguide sensors. This is a favourable approach, as unlabelled detection for OP can be achieved without the need for chromophore/fluorophore labelling, which suffers from major problems, such as being time consuming, moderately expensive, problems with dye bleaching in the case of entrapment or photobleaching in the case of using fluorophores. In addition to avoiding these problems, the hydrogel has a high percentage of water (aqueous environment), which is a good environment for preserving the enzyme activity.

The effect of the concentrations of co-monomers and cross-linkers were studied carefully before optimising the response of the pH-sensitive hydrogel for enzymatic applications.[60] It was found that using 5 mol% of EDMA and 6 mol% of MAA provided a flow-stable hydrogel (able to shrink/swell many times without collapsing) with a stable and sensitive response.

It is known that the effect of temperature, buffer composition and ionic strength affect the degree of swelling of the hydrogel containing ionisable groups. As the ionic strength increases, the swelling of the hydrogel will decrease due to the shielding effect of the counter ion on the charges on the polymer backbone. The sensors signal and response times were optimised with respect to the buffer pH, ionic concentration of buffer and temperature using paraoxon as substrate. The measurements were obtained using a sensor operated at 20 °C in 20 mM Ches (pH = 8.5, 120 mM NaCl, 2.7 mM KCl and 20 mM $CoCl_2$) to prevent depletion of cobalt from the immobilized enzyme, as it was found the $CoCl_2$ is a vital co-factor for OPH for enzyme activity.[40]

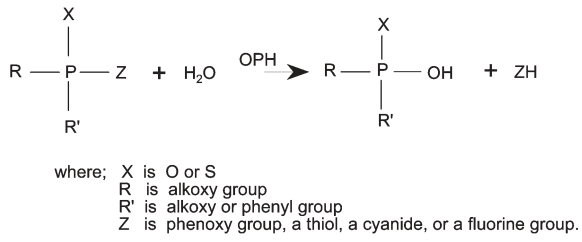

Fig. 3 Catalytic reaction of OPH.[11,62]

Operating under these conditions, the biosensor was able to detect as low as 1.5×10^{-7} M of paraoxon with very good accuracy and selectivity. Other non-organophosphate pesticides, such as simazine and triazine, did not give any signal when they were incubated with the sensor for more than 5 min. Control waveguiding with immobilized bovine serum albumin did not give any response when incubated with paraoxon. The biosensor was completely stable for at least 30 days when it was stored at 4 °C in 20 mM phosphate buffer (pH = 8.3) in 100 mM NaCl, 2.7 mM KCl, 30 mM $CoCl_2$.

Fig. 4 shows the OPH enzyme biosensor response when incubated with OP samples of different concentrations. These results revealed that after the sensor has been incubated with paraoxon, the hydrogel swelling and the resulting shift in the out-coupled angle is proportional to paraoxon concentration. There was no response when the sensor was incubated with triazine as a control analyte.

Fig. 5 shows the highly reproducible response of the sensor to a periodic exposure to 27×10^{-7} M paraoxon (RSD = 4.8%, n = 7). The calibration curve for the OPH-sensor for exposure to various concentrations of paraoxon is shown in Fig. 6.

Absorbance detection

In this part of the work, bromocresol green (BCG) acid–base indicator was entrapped within a sol-gel matrix along with the OPH enzyme. The sensor was not tested directly after preparation, but after storage at 4 °C for 20 days, because it is known that the pore structure of the sol-gel undergoes complex changes for two weeks after preparation.[61] In order to prevent densification of the pore structure during ageing,

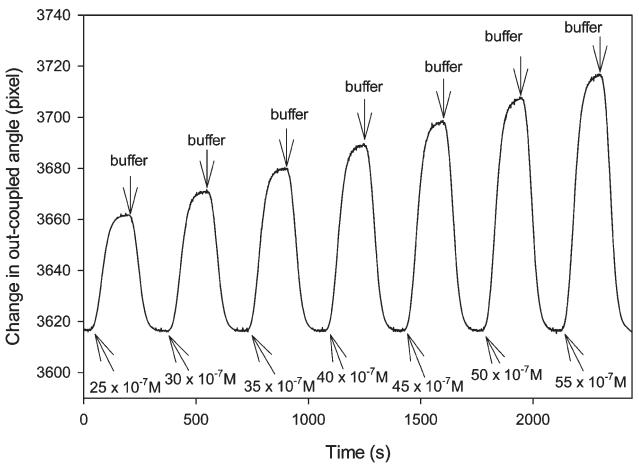

Fig. 4 The response of the TE_0 mode to different concentrations of paraoxon (data smoothed for clarity).

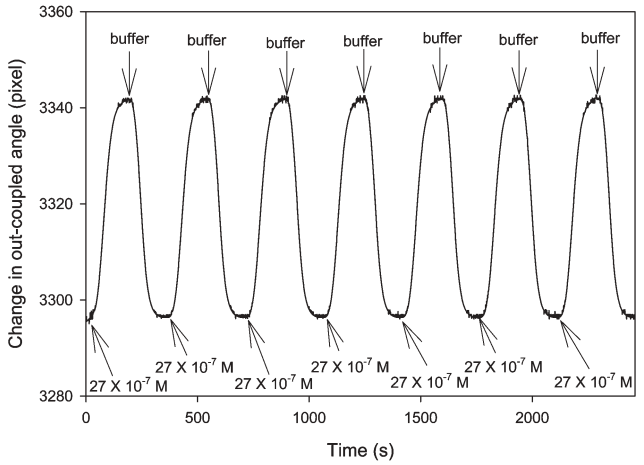

Fig. 5 Reproducibility of the TE_0 mode of the solvent blue-CLW for 27×10^{-7} M paraoxon (data smoothed for clarity).

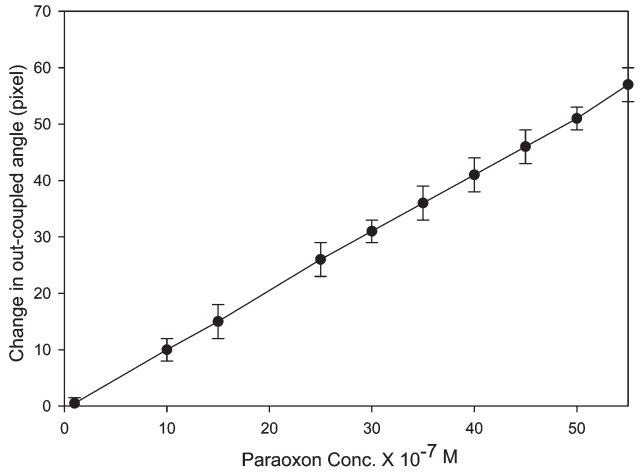

Fig. 6 Calibration curve of the paroxon TE_0 mode of the solvent blue-CLW to various concentrations of paraoxon. Error bars represent $\pm$ 1 standard deviation, $n = 5$.

which impairs the diffusion of OP into the matrix, sorbitol was added to the sol-gel. Before measurements were made the sorbitol was washed out of the matrix leaving pores that paraoxon can easily enter. Without addition of sorbitol, the apparent activity of paraoxon is approximately 8-fold lower than expected. The liberated two protons from the hydrolysis process of the OP decreased the pH in the local environment of the enzyme sensor. This pH change was apparent as a colour change of bromocresol green, leading to an increase in reflectivity of the TE_0 mode intensity when measured at 610 nm. Fig. 7 shows examples of the response of the OPH-based sensor and the calibration curve for different concentrations of paraoxon. The limit of detection was calculated to be 4×10^{-9} M for paraoxon and parathion and 8.1×10^{-8} M for diazinon. In this work this quantity has been taken as the value of the concentration that gave a signal three times the background noise. The response of the OPH-based optical sensor to a periodic exposure of 30×10^{-9} M paraoxon was reproducible (R.S.D. = 1.7%, $n = 6$). This indicates that there is no diffusion limitation of the paraoxon analyte or product

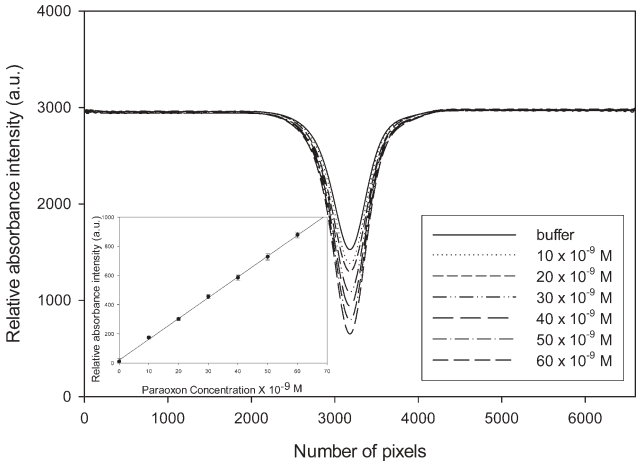

Fig. 7 The change in the TE_0 solvent blue-CLW mode intensity and the calibration curve for different concentrations of paraoxon. Error bars represent $\pm$ 1 standard deviation, $n = 4$.

accumulation, due to the increased porosity of the sol-gel caused by the sorbitol.

Fluorescence detection

The detection in this system was based on a fluorescent, pH-sensitive, fluorescein isothiocyanate (FITC) covalently immobilized with the OPH enzyme entrapped in a sol-gel. The sensing scheme is based on the measurement of the quenching of the fluorescence of the fluorescein isothiocyanate due to the liberation of protons as a result of the hydrolysis of the OP pesticides.[62] The sensor was tested after two days storage in phosphate buffer at 4 °C. In this study sorbitol was also added to the sol-gel during the preparation to accelerate the diffusion of paraoxon to the entrapped OPH within the sol-gel. Fig. 8 shows the typical response of the OPH sensor as well as the calibration curve to different concentrations of OP pesticides. The detection limit of the sensor using the OPH enzyme for paraoxon and parathion was 2.3×10^{-7} M and 9.3×10^{-6} M for diazinon. This value has been taken as the value of the concentration that gave a signal three times the background noise. The reproducibility of fluorescence quenching of the fluorescein isothiocyanate was very good (RSD = 3%, $n = 7$) due to the liberated protons for a periodic exposure of 10×10^{-7} M paraoxon.

Conclusion

Absorbing materials-clad, leaky waveguide sensors have been fabricated using solvent blue and polythiophene as clad layers and sol-gel and hydrogel as waveguiding layers. The solvent-blue devices were tested for the detection of organophosphorus pesticides by monitoring the change in refractive index when OPH enzyme immobilized within a pH-sensitive matrix. The sensitivity of the solvent blue- and polythiophene-clad platforms were also examined with regard to the detection of absorbance and fluorescence changes using an acid–base indicator, along with the OPH enzyme entrapped within a sol-gel matrix.

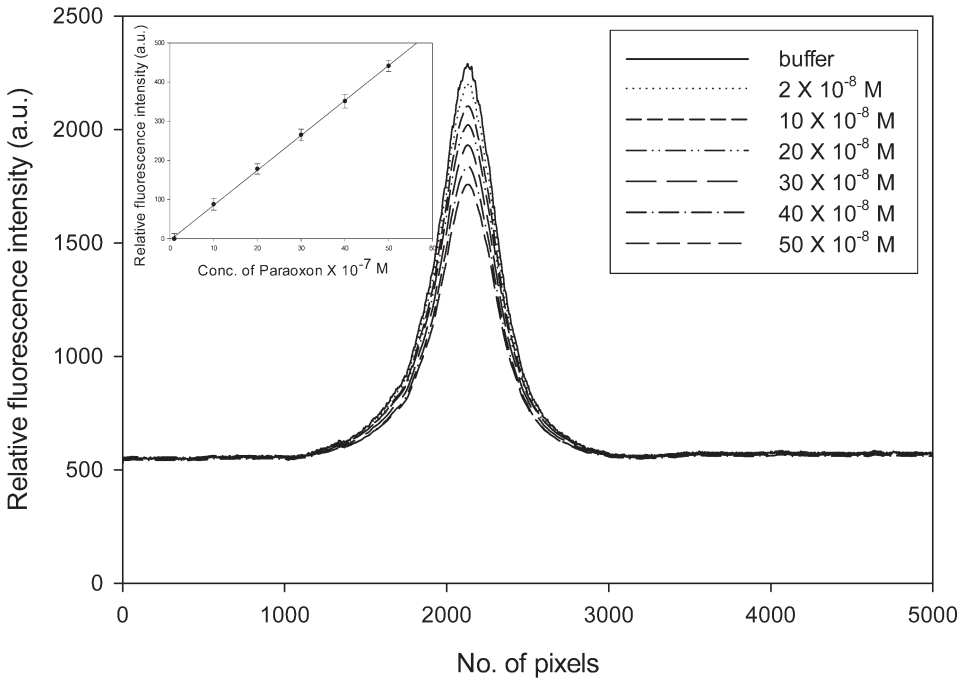

Fig. 8 The decrease in the fluorescence of FITC-labelled OPH TE_0 mode of PT-CLW and the calibration curve for different concentrations of paraoxon. Error bars represent $\pm$ 1 standard deviation, $n = 4$.

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