

# Cross-linked enzyme crystals of organophosphate hydrolase for electrochemical detection of organophosphorus compounds

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**Abstract** Cross-linked enzyme crystals of organophosphate hydrolase (CLEC-OPH) prepared from crude recombinant *E. coli* cell lysate was used for the development of an electrochemical biosensor for the detection of organophosphate pesticides. CLEC-OPH showed an increased  $V_{\max}$  of 0.721 U mg protein<sup>-1</sup> and a slightly lower  $K_m$  of 0.083 mM on paraoxon compared to the crude enzyme, resulting in an improved catalytic efficiency ( $k_{\text{cat}}/K_m = 4.17 \times 10^5 \text{ M}^{-1} \text{ min}^{-1}$ ) with a remarkable increase on thermostability. An amperometric biosensor was constructed based on glutaraldehyde and albumin cross-linkage of CLEC-OPH with carbon nanotubes. The sensor exhibited greater sensitivity and operational stability with a lower limit of detection when compared with a sensor using an equivalent loading of crude OPH in a non-crystal form. The application of crude enzyme-based CLEC would offer a simple and economical approach for the fabrication of efficient electrochemical biosensors.

**Keywords** Biosensor · Cross-linked enzyme crystal · Organophosphate hydrolase · Paraoxon

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## Introduction

Enzyme stabilization is essential for efficient biotechnological application of biocatalysts in a number of research and development fields. Among many methodologies developed for stabilization of enzymes, cross-linked enzyme crystal (CLEC) technology has recently received great attention. CLEC preparation includes crystallization of enzymes into microcrystals and subsequent cross-linking with homobifunctional or heterobifunctional reagents (Margolin and Navia 2001). Due to stabilization of the enzyme's active conformation in three-dimensional crystal structure, the microcrystalline enzymes offer many advantages, including improved thermostability, physical stability and tolerance to organic solvents, with an increase on volumetric activity (Roy and Abraham 2004). CLEC is thus considered a simple and cost-effective approach for preparation of enzymes for a range of biocatalytic applications, including organic synthesis, starch saccharification, and biosensors.

Application of CLECs in biosensor application have been reported using glucose oxidase (Mattos et al. 2001), urease (Navia and St. Clair 1999), horseradish peroxidase (Roy and Abraham 2004), and recently with laccase (Roy et al. 2005), for detection of compounds of medical and environmental importance. Compared to conventional immobilization of enzyme on electrode, CLECs exhibit increased sensitivity and stability. The application of CLECs for biosensor is thus of great interest.

Organophosphate hydrolase (OPH) is an organophosphorus hydrolyzing enzyme, first discovered in soil microorganisms, *Pseudomonas diminuta* (Dumas et al. 1989) and *Flavobacterium* sp. (Mulbry and Karns 1989b). The enzyme is specific to ester bond hydrolysis in a range of neurotoxic organophosphate pesticides, including paraoxon,

parathion, methyl-parathion, coumaphos and chemical warfare agents, sarin and soman, releasing the electroactive *p*-nitrophenol (PNP) as the product. Several types of biosensor using OPH for detection of organophosphate compounds have been developed, based on optical (Mulchandani et al. 1999b; Rogers et al. 1999; Simonian et al. 2005), potentiometric (Mulchandani et al. 1999c), and amperometric (Mulchandani et al. 2001; Chough et al. 2002; Wang et al. 2003; Deo et al. 2005) detection. However, the commercial application of OPH-biosensor has been impeded by several factors, including high cost on enzyme production, purification and processing.

In this work, the simple preparation and kinetic study of the crude enzyme-based CLECs is described. Application of CLEC-OPH was compared for analytical utility to the conventional cross-linked form in amperometric detection of organophosphorus compounds. This report demonstrates the potential of using crude enzyme-based CLECs as cost-effective biocatalysts for further development of efficient electrochemical biosensors.

## Materials and methods

### Reagents and chemicals

Ampicillin, chloramphenicol and glutaraldehyde were obtained from Fluka. Isopropyl  $\beta$ -D-1-thiogalactopyranoside (IPTG) and DNaseI were purchased from Fermentas (Vilnius, Lithuania). Phenylmethane sulfonyl fluoride (PMSF) was supplied from Bio-Rad (Hercules, CA). Bovine serum albumin (BSA) was obtained from Sigma. Paraoxon was acquired from Supelco (Bellefonte, Pennsylvania). Solvents were analytical grade including isopropanol (Merck, Whitehouse Station, NJ) and *N,N*-dimethylformamide (DMF) (Ajax Finechem, Seven Hills, Australia). Multi-walled carbon nanotubes (MWNTs) was purchased from MER Corporation (Tucson, Arizona). All chemicals were used as received without further purification. All solutions were made in double distilled water.

### Preparation of crude recombinant OPH

The 1.1-kbs *opd* gene was amplified from the indigenous plasmid of *Flavobacterium* sp. ATCC27551 and cloned into pET-32a (Novagen, Darmstadt, Germany) at the *Nco*I and *Xho*I sites. The resultant plasmid, pTOPD, was transformed into a protease deficient host, *E. coli* Rosetta(DE3)(pLysS) (Novagen) for recombinant enzyme expression. The recombinant *E. coli* was grown in Luria Bertani broth (LB) (1% tryptone, 0.5% yeast extract, and 1% NaCl), supplemented with 100  $\mu$ g/ml ampicillin and 25  $\mu$ g/ml chloramphenicol for overnight with shaking at 37°C. One

percent (v/v) culture was inoculated into a fresh medium with the same composition and incubation condition. When the cell reached the mid-log phase ( $OD_{600} = 0.5$ – $0.6$ ), 0.5 mM IPTG (final concentration) was added to induce the recombinant enzyme expression, and 50  $\mu$ M  $CoCl_2$  was used as a supplement. The cells were cultured in an orbital shaker at 18°C, 200 rpm for 8 h. Cell pellet was harvested by centrifugation at  $9,000 \times g$  for 10 min, and then resuspended in a lysis buffer (50 mM Tris-HCl (pH 8.0), 50  $\mu$ M  $CoCl_2$ , 2.5% Triton X-100, 5 mM PMSF and 10  $\mu$ g/ml DNaseI). The cell suspension was cooled on ice and passed through a French Pressure cell (1,500 psi). The cell lysate was clarified by centrifugation at  $9,000 \times g$ , 4°C for 10 min. The supernatant containing OPH activity was concentrated and transferred to 50 mM Tris-HCl buffer (pH 8.0) by ultrafiltration using Amicon ultrafiltration unit (10 kDa MWCO) (Millipore, Billerica, MA) and used as the crude OPH for further experiments.

### Crystallization of OPH

Crude OPH was crystallized by batch method based on the condition previously reported (Roy and Abraham 2006). Ammonium sulfate in small portions was added to the crude OPH (20 mg/ml) in 50 mM Tris-HCl buffer (pH 8.0) to reach 75% saturation over 3 h by stirring at 4°C. The supersaturated solution was kept at this temperature, undisturbed for 20 h. The enzyme crystals formed were separated by centrifugation at  $500 \times g$  for 8 min and washed with isopropanol to remove the excess ammonium sulfate. For cross-linking reaction, 20 mg of OPH crystals was reacted with 2 ml of 1.5% v/v glutaraldehyde solution in isopropanol for 20 min at room temperature. After cross-linking, the enzyme crystals (CLEC-OPH) were washed three times with 50 mM Tris-HCl (pH 8.0) to remove the excess glutaraldehyde and stored in the same buffer for further experiments.

### Spectroscopic activity assay of soluble OPH

Organophosphate hydrolase activity of the soluble crude OPH was measured spectrophotometrically by monitoring the production of *p*-nitrophenolate. The standard assay reactions of 1 ml contained 0.5 mM paraoxon in 100 mM sodium phosphate buffer (pH 8.0), and an appropriate dilution of OPH was added to start the reaction. The assay reaction was incubated at 25°C. The rate of *p*-nitrophenolate formation was calculated from the measurement of  $OD_{400}$  at time intervals in the period of 3 min ( $\epsilon = 17,000 \text{ M}^{-1} \text{ cm}^{-1}$ ). One unit of enzymatic activity was defined as the amount of enzyme capable to catalyzing the production of 1  $\mu$ mol of *p*-nitrophenolate per minute under the standard conditions.

### Spectroscopic activity assay of CLEC-OPH

The assay reaction of 1-ml total volume contained 0.25 mM paraoxon in 100 mM sodium phosphate buffer (pH 8.0). CLEC-OPH ( $1.22 \times 10^{-3}$  U) was added to the assay reaction and stirred continuously for 5 min. The increase in absorbance was monitored at 30 s and at definite time intervals at 400 nm.

### Kinetic parameter analysis

Kinetic parameters of the crude soluble OPH and CLEC-OPH were determined under the standard assay conditions with varying paraoxon concentration, while keeping the concentration of the biocatalyst constant. The Michaelis–Menten constant ( $K_m$ ) and maximum velocity ( $V_{max}$ ) were calculated from the data obtained fitted by linear least square regression.

### Determination of protein

The concentration of protein in the soluble crude enzyme and CLEC was determined using Lowry's method according to Abraham et al. 2004 (Abraham et al. 2004; Rajan and Abraham 2007). Bovine serum albumin (BSA) was used as the standard.

### Preparation of OPH electrodes

MWNTs functionalized with carboxylic acid groups were dispersed in DMF and sonicated for 30 min. A volume of 13.5  $\mu$ l consisting of 8  $\mu$ l of the enzyme solution or suspension (containing  $1.22 \times 10^{-3}$  U per electrode of either crude enzyme or CLEC-OPH), 2  $\mu$ l of MWNTs suspension, 2  $\mu$ l of 1 mg/ml BSA and 1.5  $\mu$ l of 2% (v/v) glutaraldehyde solution, was deposited on a glassy carbon electrode after the components were mixed to homogeneity. The coating was air dried at room temperature.

### Apparatus

Electrochemical measurements were performed using an Autolab PGSTAT12 Electrochemical Analyzer (EcoChemie, Utrecht, The Netherlands). The working electrode was glassy carbon (3.0 mm diameter) (BAS, Tokyo, Japan). Before each experiment, the working electrode was polished with an alumina-water slurry on cotton wool and then sonicated in distilled water. Potentials were quoted relative to the Ag/AgCl reference (BAS). A platinum disk (5.0 mm diameter) was used as the counter. All electrochemical experiments were performed in 100 mM sodium phosphate buffer (pH 8.0), unless otherwise stated.

## Results and discussion

Recombinant expression of OPH usually results in low active enzyme yield due to low solubility of this protein in *E. coli* (Mulbry and Karns 1989a; Grimsley et al. 1997). Though several approaches have been investigated for improved production of OPH in recombinant systems, including multi-gene fusions (Wu et al. 2001), fusion with highly-soluble fusion partners (Cha et al. 2000), display on cell surface (Richins et al. 1997; Li et al. 2004), and periplasmic secretion (Kang et al. 2005), reaching high productivity of OPH using *E. coli*-based fermentation or in other recombinant systems (Takayama et al. 2006) is still challenging, especially for further cost-efficient production of highly purified enzymes for electrochemical biosensor applications. Finding a simple alternative approach for OPH preparation in biosensor systems is thus of great interest.

In this work, recombinant OPH was predominantly expressed in inclusion form with minor fraction in active soluble form under the experimental conditions. Western blot analysis using anti-His<sub>6</sub> antibodies showed a specific band corresponding to OPH in the soluble protein fraction of the recombinant *E. coli* (data not shown). The low expression yield of OPH was similar to some previous works. Nevertheless, as no background hydrolysis of organophosphate compounds was detected in the crude cell lysate of control *E. coli* carrying pET-32a without *opd*, this paved the way for the direct use of the crude enzyme from recombinant *E. coli* expressing OPH for CLEC preparation.

Cross-linked enzyme crystal technique offers a simple and economical approach for preparation of biocatalyst for biosensor application. In almost all cases, CLECs have been prepared from enzyme with high purity, mostly for organic synthesis application with very few exceptional cases where the crystalline enzymes were prepared from crude industrial enzymes (Abraham et al. 2004; Dalal et al. 2007).

### Characterization of CLEC-OPH

In this work, for the first time, the catalytically active CLEC-OPH was prepared from crystallization of the crude recombinant *E. coli* cell lysate as a simple enzyme immobilization approach. OPH was crystallized by batch crystallization method. The concentration of glutaraldehyde at 1.5% for cross-linking gave catalytically active enzyme crystals. The general properties of CLEC-OPH in comparison to the crude OPH are given in Table 1. The specific activity of CLEC-OPH was 4-times higher than that of the crude soluble enzyme. This might be due to partial purification of the enzyme and possibly separation

**Table 1** General properties of CLEC-OPH

Property	CLEC-OPH	Crude OPH
Specific activity (U <sup>a</sup> mg protein <sup>-1</sup> )	0.542	0.136
$K_m$ (mM)	0.083	0.111
$V_{max}$ (U mg <sup>-1</sup> )	0.721	0.168
$k_{cat}$ (min <sup>-1</sup> )	34.61	8.07
$k_{cat}/K_m$ (M <sup>-1</sup> min <sup>-1</sup> )	$4.17 \times 10^5$	$9.72 \times 10^4$

<sup>a</sup> U defined as  $\mu\text{mol min}^{-1}$ ; using paraoxon as substrate for pesticide hydrolysis by OPH

of inhibitory substances in the ammonium sulfate precipitation step.

Kinetic parameters were determined using the Michaelis-Menten equation from a Hanes-Woolf plot. For CLEC-OPH, the Michaelis-Menten constant  $K_m$  was 0.083 mM ( $\pm 0.0056$  SD,  $n = 3$ ) and the maximum velocity  $V_{max}$  was 0.721 U mg<sup>-1</sup> crystal protein ( $\pm 0.0344$  SD,  $n = 3$ ). Compared to the crude soluble enzyme, the crystalline enzyme had increased  $V_{max}$  and slightly higher affinity to the substrate. This led to an improved catalytic constant  $k_{cat}$  and hence, a 4.3-fold increase in catalytic efficiency,  $k_{cat}/K_m$ . Typically, immobilized enzymes exhibit an increased  $K_m$  relative to the solution value. However, the apparent  $K_m$  obtained from CLEC-OPH was not significantly increased. This agrees with results obtained from CLEC formed from pancreatic acetone powder (Dalal et al. 2006) and pectinase, xylanase and cellulase (Dalal et al. 2007). This was attributed by the authors to the enzyme aggregation in CLEC and to the presence of an open structure in which mass transport of substrate was similar to that at the soluble enzyme, when low concentrations of glutaraldehyde were used (Dalal et al. 2007). Meanwhile,  $V_{max}$  for CLEC-OPH was far greater than for the soluble form. This is probably due to partial purification of the enzyme by protein fractionation in the precipitation step resulting in higher enzyme specific activity. This has also been observed in the case of CLEC containing pectinase,

xylanase and cellulase (Dalal et al. 2007) and glucoamylase (Abraham et al. 2004). Hence, the catalytic enzyme rate constant  $k_{cat}$  and the catalytic efficiency, which is related to  $V_{max}$ , were higher than in the soluble crude OPH.

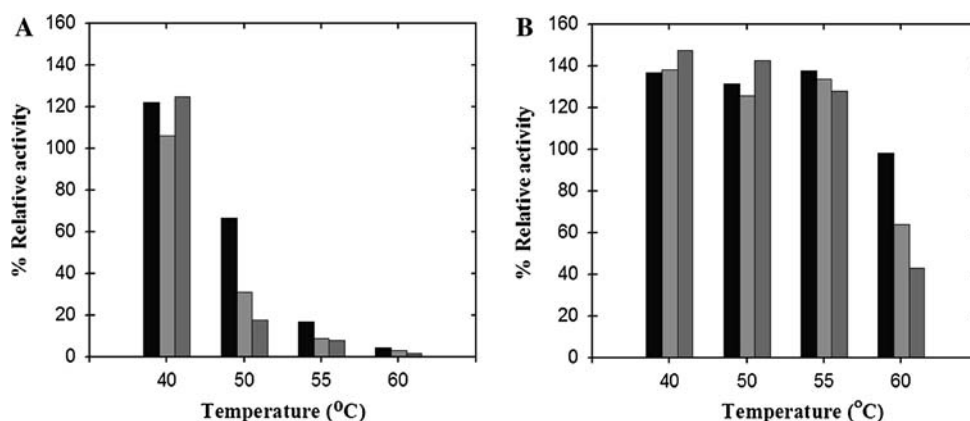
Thermostability of CLEC-OPH was remarkably improved when compared to the soluble enzyme (Fig. 1). Pre-incubation of CLEC-OPH and crude OPH at 40°C led to activation of the enzyme activity, with 147% activity increase observed for CLEC-OPH after incubation for 4 h. At higher temperatures, from 50°C, the thermal inactivation of the crude enzyme became more pronounced and complete loss of activity was observed after incubation at 60°C for 1 h. In contrast to the crude enzyme, CLEC-OPH retained full activity at temperature up to 55°C. Gradual inactivation of the microcrystalline enzyme was observed at 60°C and 43% of the catalytic activity was retained after incubation for 4 h. Activation of CLEC's catalytic activity by pre-incubation at elevated temperatures was previously reported in microcrystalline glucoamylase (Abraham et al. 2004). The remarkable enhancement of thermostability in CLEC-OPH would be due to stabilization of the catalytically active conformation of the enzyme by chemical crosslinking of OPH with the heterogeneous proteins in the crystal lattice, resulted from the inter- and intramolecular interactions which led to increased rigidity of the three-dimensional conformation of molecules in CLECs (Roy and Abraham 2004).

### Application of CLEC-OPH in electrochemistry

#### Optimization of CLEC-OPH amperometric biosensor

As the first step in CLEC application on electrochemical biosensor, the detection conditions and parameters were optimized. Using the electrode modification procedure described in the experimental section, we examined the effect on the electrode response to varying mass of CLEC-OPH. The optimum CLEC-OPH-loading was found to be

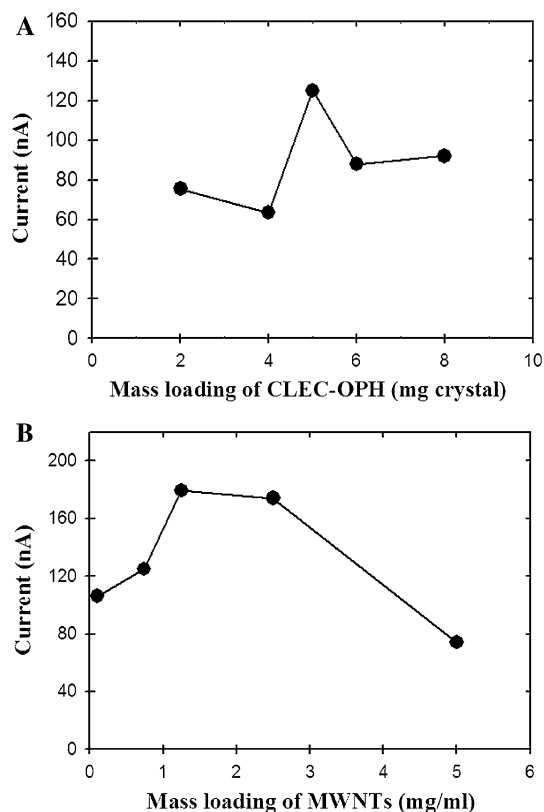
**Fig. 1** Thermostability of crude OPH (a) and CLEC-OPH (b) at 40–60°C for (■) 1 h, (▒) 2 h, and (■) 4 h



5 mg crystal per electrode (Fig. 2a). Increasing the crystal beyond 5 mg resulted in a decreased paraoxon signal, presumably because the increased density of CLEC caused a barrier for diffusion of *p*-nitrophenol to the electrode.

MWNTs were used to promote the oxidation of phenolic compounds and to minimize surface fouling (Wang 2005; Balasubramanian and Burghard 2006). The MWNTs were co-immobilized with the CLEC-OPH based on previous reports in which CNTs incorporated with adsorbed enzyme on an electrode resulted in enhanced sensitivity (Joshi et al. 2005). The mass of the MWNTs incorporated was also examined. The sensitivity increased slightly upon raising the MWNTs loading between 0.10 and 1.25 mg/ml and decreased rapidly thereafter (Fig. 2b). The initial increase in MWNTs represents an increased surface area for *p*-nitrophenol reaction, whereas a very high mass of MWNTs may obstruct analyte diffusion to the enzyme.

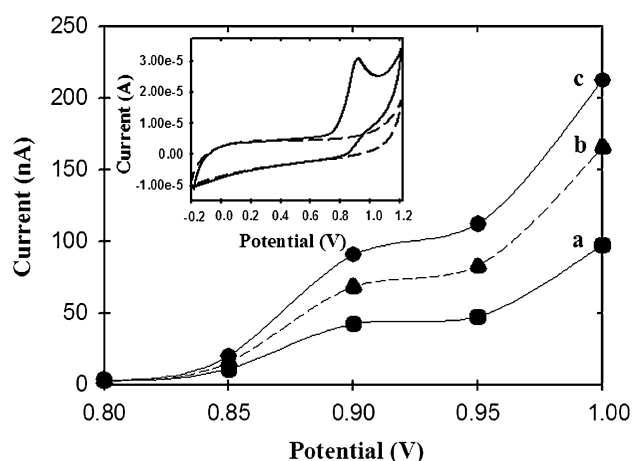
To determine the appropriate potential for *p*-nitrophenol oxidation, hydrodynamic voltammetry was performed. Figure 3 shows the current response of the electrode using different concentrations of paraoxon at different applied potentials. It can be seen that the current responses were independent to potential between 0.90 and 0.95 V, and then at >0.95 V rising currents were observed. This was unlikely to be due to water hydrolysis since no gas bubbles



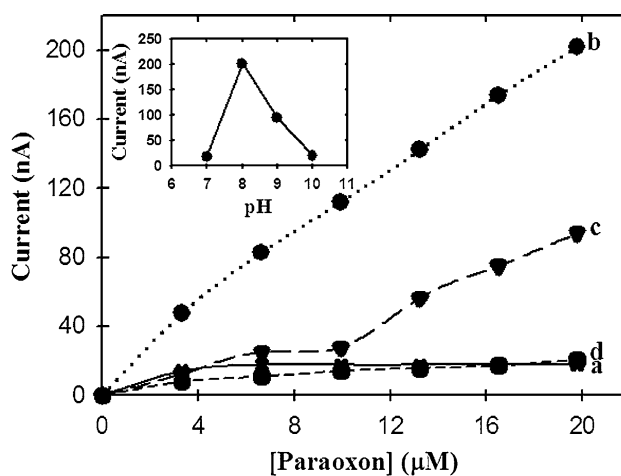
**Fig. 2** Amperometric current at 0.95 V versus Ag/AgCl to 19.77 μM paraoxon at varying amount of CLEC-OPH (a) and MWNT (b)

were observed. It may be due to the direct electrochemical oxidation of paraoxon, which has been reported previously at approximately this potential (Mulchandani et al. 1999a). On the basis of this result, the applied potential was set at 0.95 V for further work.

The effect of pH on the response of the CLEC-OPH electrode to paraoxon was evaluated in 100 mM sodium phosphate buffer (pH 7.0–pH 8.0) and 100 mM glycine-NaOH (pH 9.0–pH 10.0) (Fig. 4). The electrode performed best at pH 8.0. This is a similar result to previous reports examining immobilized OPH in the range pH 7.0–pH 8.5 (Mulchandani et al. 1999a; Chough et al. 2002; Wang et al. 2003; Deo et al. 2005).



**Fig. 3** Hydrodynamic voltammograms of paraoxon at (a) 3.32 μM (b) 6.63 μM and (c) 9.93 μM at a CLEC-OPH electrode. Inset: Cyclic voltammogram at a scan rate of 0.1 V s<sup>-1</sup> in the absence (—) and presence of 1 mM *p*-nitrophenol (---)



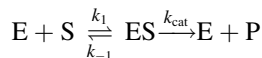
**Fig. 4** Effect of pH on amperometric response of the CLEC-OPH electrode at 0.95 V versus Ag/AgCl in sodium phosphate buffer at (a) pH 7.0, and (b) pH 8.0, and glycine-NaOH buffer at (c) pH 9.0, and (d) pH 10.0. The inset shows pH against the responses

### Analytical characterization of CLEC-OPH biosensor

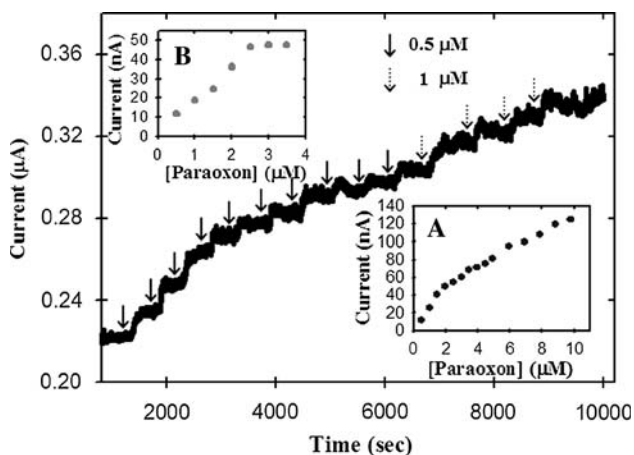
An electrode modified with the OPH crystal was compared to one modified with the non-crystal form. The latter was immobilized by glutaraldehyde cross-linking of the crude OPH under the same conditions and enzyme unit activity. Figure 5 in inset A displays calibration plots for paraoxon at the CLEC-OPH electrode, and the crude OPH electrode is illustrated in inset B. The sensitivity for the CLEC-OPH electrode over the 0.5–2.5  $\mu\text{M}$  range was 25.95 nA/ $\mu\text{M}$ , which is ca. 1.5-fold greater than the crude OPH electrode (Table 2). Partition of paraoxon into the two enzyme films can be compared by noting the apparent  $K_m$  values. As expected,  $K_m$  for the CLEC-OPH electrode was slightly higher than for the crude OPH film (6.19  $\mu\text{M}$  cf. 5.16  $\mu\text{M}$ ), suggesting a lower degree of substrate partition into the crystal film.

Note the immobilized CLEC-OPH and crude OPH electrodes exhibited far lower  $K_m$  values than the enzyme in bulk solution ( $K_m$  evaluated from absorbance of *p*-nitrophenolate). This result may indicate a high substrate concentration close to the enzyme electrode due to

favorable substrate adsorption to either the CLEC/crude enzyme surface or to the electrode surface. For the general enzyme process:



$K_m$  is defined as  $(k_{-1} + k_{\text{cat}})/k_1$ . Hence, if the substrate concentration at the enzyme is higher than the substrate concentration in bulk solution the rate of the reaction  $E + S \rightarrow ES$  will be increased, giving a larger value of  $k_1$ . Therefore the value of  $K_m$  will be lower. The value  $i_{\text{max}}$  is proportional to the maximum reaction rate ( $V_{\text{max}} = k_{\text{cat}} E_T$ ). From the experiment,  $i_{\text{max}}$  for the CLEC-OPH electrode was greater than the crude OPH (189.23 nA cf. 127.04 nA), despite a lower degree of partitioning. This emphasizes the high catalytic activity provided by the crystal lattice. The limit of detection (3 times the standard deviation of the response obtained for a blank) was 0.314  $\mu\text{M}$  at the CLEC-OPH electrode, and 0.5  $\mu\text{M}$  for the cross-linked OPH electrode. For repeated uses in batch a measurement, the sensor response of the CLEC-OPH based electrode decreased gradually after sixteen successive assays of 0.5 and 1  $\mu\text{M}$  paraoxon (Fig. 5) compared to that of the electrode using crude OPH of which the sensor response stopped after five assays. The result thus, indicated the higher operational stability of the CLEC-based biosensor. The marked improvement in operational stability of CLEC-OPH, compared to the soluble enzyme would be related to the more extensive interactions in stabilization of enzyme's active conformation in the crystalline structure by intra-/intermolecular interactions (Roy and Abraham 2004). The result would thus indicate an additional advantage of CLECs in electrochemical biosensor application. The biocatalytic activity of CLEC-OPH was maintained over a prolonged period of at least 3 months (keeping at 10°C), reflecting the storage stability of the enzyme.



**Fig. 5** Amperometric responses of paraoxon at a CLEC-OPH electrode. Arrows refer to adding points. Inset A: Calibration at the CLEC-OPH electrode and inset B represents the crude OPH electrode. Both electrode responses were obtained by using  $1.22 \times 10^{-3}$  U of enzyme

**Table 2** Electrochemical detection performance of CLEC-OPH electrode and crude-OPH electrode

Property	CLEC-OPH electrode	Crude OPH electrode
Sensitivity (nA $\mu\text{M}^{-1}$ )	25.95	17.29
Linear range ( $\mu\text{M}$ )	0.50–2.00	0.50–2.50
Detection limit ( $\mu\text{M}$ )	0.314	0.500

### Conclusions

In conclusion, an amperometric biosensor for paraoxon has been developed by the use of crude enzyme-based CLECs, which provide a simple route to constructing biosensors using crude rather than the costly purified OPH. The resulting biosensor exhibited a sensitivity similar to some previous reports on paraoxon measurement by amperometric biosensors using purified OPH, including the one of the highest reported sensitivity (25.95 nA/ $\mu\text{M}$  cf. 25 nA/ $\mu\text{M}$ ) but showed a slightly higher limit of detection (0.314  $\mu\text{M}$  cf. 0.150  $\mu\text{M}$ ) (Deo et al. 2005). The developed CLEC-OPH biosensor could possibly be improved by lowering the electrical noise of the hardware. The use of the crude enzyme in CLEC form may thus lead to the development of

OPH-based biosensors of an economically viable production cost.

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