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Mussel adhesive protein-based whole cell array biosensor for detection of organophosphorus compounds

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ABSTRACT

A whole cell array biosensor for the efficient detection of neurotoxic organophosphate compounds (OPs) was developed through the immobilization of recombinant *Escherichia coli* cells containing periplasmic-expressing organophosphorus hydrolase (OPH) onto the surface of a 96-well microplate using mussel adhesive protein (MAP) as a microbial cell-immobilizing linker. Both the paraoxon-hydrolyzing activity and fluorescence microscopy analyses demonstrated that the use of MAP in a whole cell biosensor increased the cell-immobilizing efficiency and enhanced the stability of immobilized cells compared to a simple physical adsorption-based whole cell system. Scanning electron microscopic analyses also showed that the *E. coli* cells were effectively immobilized on the MAP-coated surface without any pretreatment steps. The whole cell array biosensor system, prepared using optimal MAP coating (50 $\mu g/cm^2$) and cell loading (4 OD_{600}), detected paraoxon levels as low as 5 μM with high reproducibility, and its quantitative detection range was ~5–320 μM. The MAP-based whole cell array biosensor showed a good long-term stability for 28 day with 80% retained activity and a reusability of up to 20 times. In addition, paraoxon in tap water was also successfully detected without a reduction in sensitivity. Our results indicate that the proposed MAP-based whole cell array system could be used as a potential platform for a stable and reusable whole cell biosensor.

1. Introduction

Enzymes are often used as biocatalysts in biosensors due to their high specific activity (Inaba et al., 2003; Jamal et al., 2010; Park et al., 2011; Savizi et al., 2012). Whole cell biocatalysts offer several advantages over free enzymes, including high stability, reduced purification requirements, low preparation cost, and efficient cofactor regeneration (de Carvalho, 2011). Whole cell-based biosensors are used for high-throughput drug discovery, clinical diagnosis, and environmental monitoring of chemicals and heavy metals (Kumar and D'Souza, 2011a; Liu et al., 2007, 2011; Mulchandani et al., 2001; Perdikaris et al., 2011). As a key factor in the development of a whole cell biosensor, the surface immobilization technique can affect both sensitivity and stability (Alvarez et al., 2009; Braschler et al., 2005; Jen et al., 1996; Jha et al., 2009; Kumar and D'Souza, 2010, 2011a, 2011b). Physical (entrapment or adsorption) and chemical (crosslinking) methods have been widely used for surface immobilization of cells (D'Souza, 2001). Physical immobilization methods, in particular, have several limitations. The mass transfer problems occur when cells are entrapped using polymer gels, such as polyacrylamide, alginate, and agarose (Braschler et al., 2005; Jen et al., 1996), and cell immobilization using direct adsorption or adhesion may lead to the loss of cells from surface during continuous use (D'Souza, 2001). While chemical crosslinking methods can overcome the limitations of physical adsorption/adhesion (Kumar and D'Souza, 2010, 2011a, 2011b), cell viability may be negatively affected (D'Souza, 2001; Lei et al., 2006). Thus, improved immobilization techniques with both biocompatibility and strong surface adhesion would assist in the development of whole cell biosensors.

Mussel adhesive proteins (MAPs) from marine mussels are water-insoluble and biocompatible bioadhesives that can adhere tightly to substrata (Cha et al., 2008; Dove and Sheridan, 1986; Waite and Tanzer, 1981). Because MAPs are able to form strong bonds with both biomolecules and diverse surfaces including glass, metal, and plastics without any pretreatment processes (Burzio et al., 1989; Waite, 1987), they have been suggested as immobilizing agents for enzymes, proteins, and antibodies (Burzio et al., 1996; Kim et al., 2011; Saby and Luong, 1998). Our previous work on recombinant MAP in *Escherichia coli* demonstrated that recombinant MAP has both efficient adhesion ability and biocompatibility for various cell types, including mammalian and insect cells (Choi et al., 2010; Hwang et al., 2007a, 2007b, 2007c).

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Organophosphate compounds (OPs) are widely employed as pesticides and insecticides for both agriculture and household use due to their broad-spectrum activities and low cost. However, the overuse of OPs has caused environmental pollution, increasing ecological and human health risks. Some OPs have been also used in chemical warfare as neurotoxins (Donarski et al., 1989). Due to the environmental risk associated with OPs, sensitive, reliable, and cost-effective analytical tools are required to detect toxic OPs. Organophosphorus hydrolase (OPH) is a metalloenzyme produced by the soil microorganisms *Pseudomonas diminuta* and *Flavobacterium* sp. and degrades a broad spectrum of OPs (Grimsley et al., 1997; Lai et al., 1995; McDaniel et al., 1988; Mulbry and Karns, 1989). OPH has been used for the detection of OPs in the environment (Chough et al., 2002; Hossain et al., 2011a, 2011b; Mulchandani et al., 1999; Rogers et al., 1999). Several biosensors based on OPH-expressing cells have also been developed for OP detection (Kumar et al., 2006, Kumar and D'Souza, 2010, 2011a, 2011b; Mulchandani et al., 1998a, 1998b, 1998c, 2001; Rainina et al., 1996).

In this present work, we developed a whole cell array biosensor for the detection of neurotoxic OPs using MAP as a cell-immobilizing linker. We employed the previously engineered *E. coli* as a target whole cell, which expresses OPH in its periplasmic space under the assistance of chaperone to reduce substrate/product diffusion limitation and to increase OP degradation efficiency (Kang et al., 2012). Diverse aspects of the whole cell array biosensor system were evaluated, including quantitative detection limits, reproducibility, long-term stability, and reusability.

2. Experimental

2.1. Cell culture condition

The recombinant *E. coli* cells expressing OPH in the periplasmic space and GroEL/ES in the cytoplasm (Kang et al., 2012) were cultured in Luria-Bertani (LB) medium (0.5% (w/v) yeast extract, 1% (w/v) tryptophan, and 1% (w/v) NaCl) containing 50 μg/mL of ampicillin (Sigma-Aldrich, St. Louis, MO, USA) and 25 μg/mL of chloramphenicol (Sigma-Aldrich). When the culture reached a cell density (OD_{600}) of 1.2, 1 mM (final concentration) isopropyl-β-D-thiogalactopyranoside (IPTG; Sigma-Aldrich), 10 ng/mL tetracycline, and 0.5 mM $CoCl_2$ were added to induce the expression of recombinant OPH and GroEL/ES. The recombinant cells were grown at 37 °C for an additional 12 h. DsRed-expressing recombinant *E. coli* culture was grown to 0.8–0.9 OD_{600} at 37 °C in shake flask containing 50 mL LB medium with 50 μg/mL ampicillin. Protein expression was induced by adding IPTG to a final concentration of 1 mM. The recombinant cells were grown at 37 °C for an additional 10 h. The cells were harvested by centrifugation at 4000 *g* for 10 min

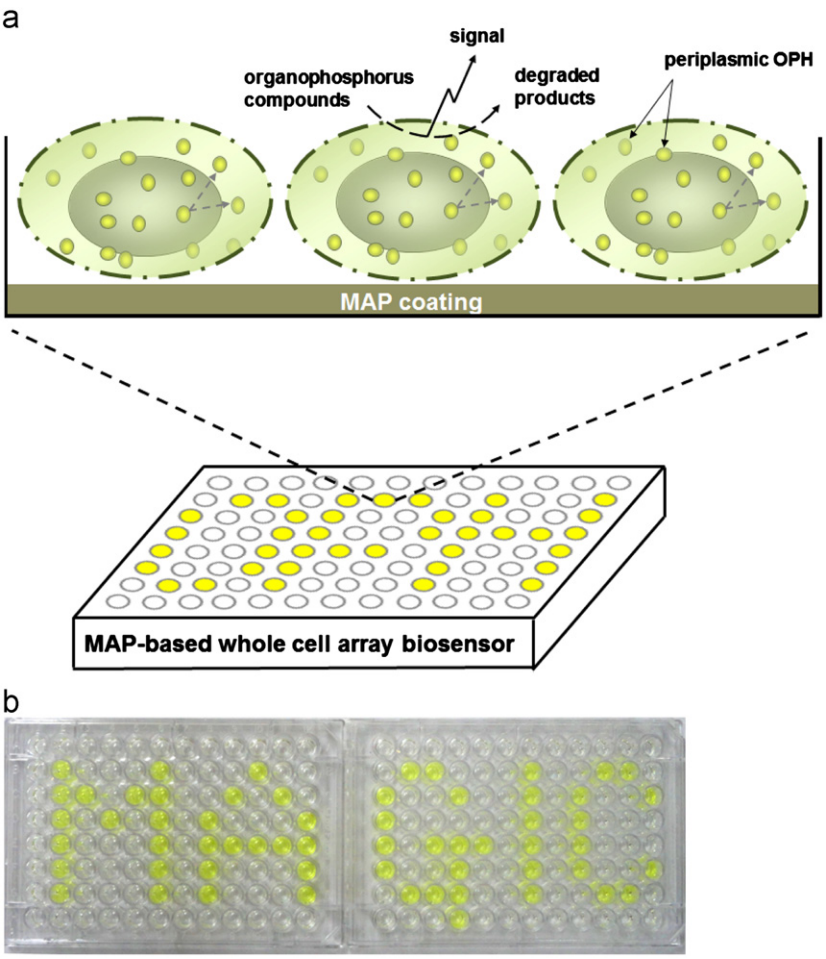

Fig. 1. (a) Schematic representation of the whole cell array biosensor for the detection of OPs and (b) demonstration of paraoxon detection as the word 'MAGIC'. Surfaces of the 96-well microplate were coated with MAP and then, recombinant *E. coli* cells expressing OPH in the periplasmic space were immobilized onto the MAP-coated well surfaces.

and resuspended in phosphate buffered saline (PBS, pH 8.0; Thermo Scientific, San Jose, CA, USA). Resuspended cells were stored at 4 °C until use.

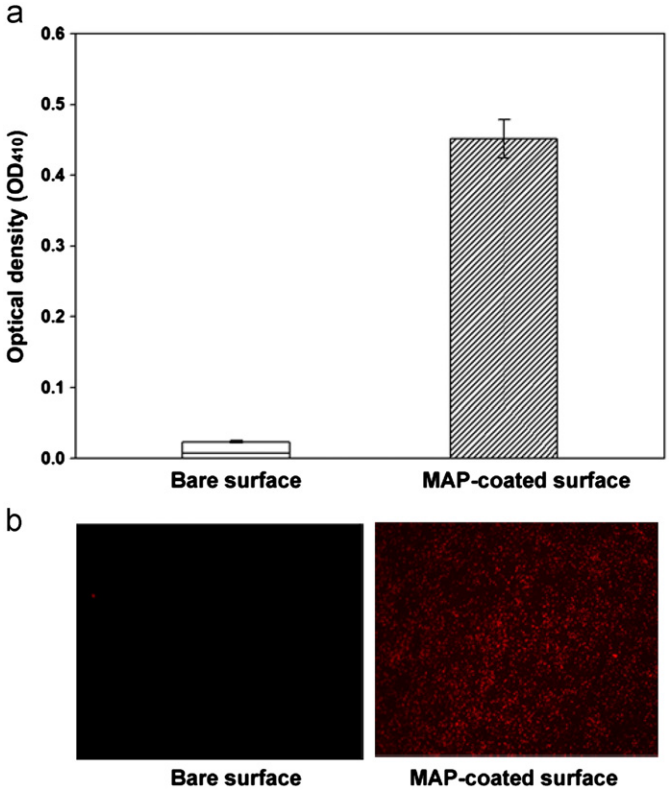

Fig. 2. (a) Paraoxon-hydrolyzing activities of OPH-expressing *E. coli* cells and (b) fluorescence images of DsRed-expressing *E. coli* cells for both bare and MAP-coated surfaces. Recombinant *E. coli* cells (4 OD_{600}) were incubated on surfaces at 37 °C for 1 h. The paraoxon hydrolysis reaction was performed at room temperature for 5 min and analyzed by measuring the absorbance at the λ_{max} of 410 nm. Each value represents the mean of three independent wells, and the error bar is the standard deviation.

2.2. Production and purification of MAP

Recombinant MAP (fp-151) was produced in *E. coli* as described previously (Hwang et al., 2007b). Briefly, cells were grown in 3 L of LB media supplemented with 50 μg/mL ampicillin in a 10-L bioreactor (KoBiotech, Incheon, Korea) at 37 °C and 250 rpm. When the culture reached an OD_{600} of 0.2–0.5, 1 mM (final concentration) IPTG was added to the culture broth to induce MAP expression. The culture broth was centrifuged at 18,000 *g* and 4 °C for 10 min. Harvested cell pellet was resuspended in 5 mL lysis buffer (10 mM Tris–HCl and 100 mM sodium phosphate, pH 8.0) per gram wet weight and lysed with constant cell-disruption systems (Constant Systems, Northants, England) at 20 kpsi. Lysate was centrifuged at 18,000 *g* and 4 °C for 20 min. The cell lysate pellet was then collected for purification and resuspended in 25% (v/v) acetic acid to extract MAP. The extraction solution was centrifuged at 18,000 *g* and 4 °C for 2 min. The supernatant was collected and freeze dried.

2.3. Immobilization of recombinant cells on MAP-coated microplate

MAP (50–500 μg/cm^2) was coated onto vacuum gas plasma-treated polystylene 96-well microplate (BD Bioscience, San Jose, CA, USA) as described in our previous work (Choi et al., 2010). MAP dissolved in 5% acetic acid was precipitated onto the 96-well microplate surface by the addition of 0.1 M sodium carbonate (pH 9.5). The coated MAP was incubated at 37 °C for 1 h. After washing with PBS buffer (pH 8.0), recombinant cells (1–32 OD_{600}) were incubated on the MAP-coated 96-well microplate at 37 °C for 1 h. The whole cell-immobilized microplate was stored at 4 °C until further use. As a control, recombinant cells were also adsorbed onto bare 96-well microplate. Morphological analyses of both MAP-coated surfaces and immobilized cells on MAP-coated surfaces were performed using scanning electron microscopy (SEM; Hitachi, Ibaraki, Japan).

2.4. Analytic performances

An analysis of the quantitative detection for the constructed whole cell array biosensor was performed using varying concentrations of

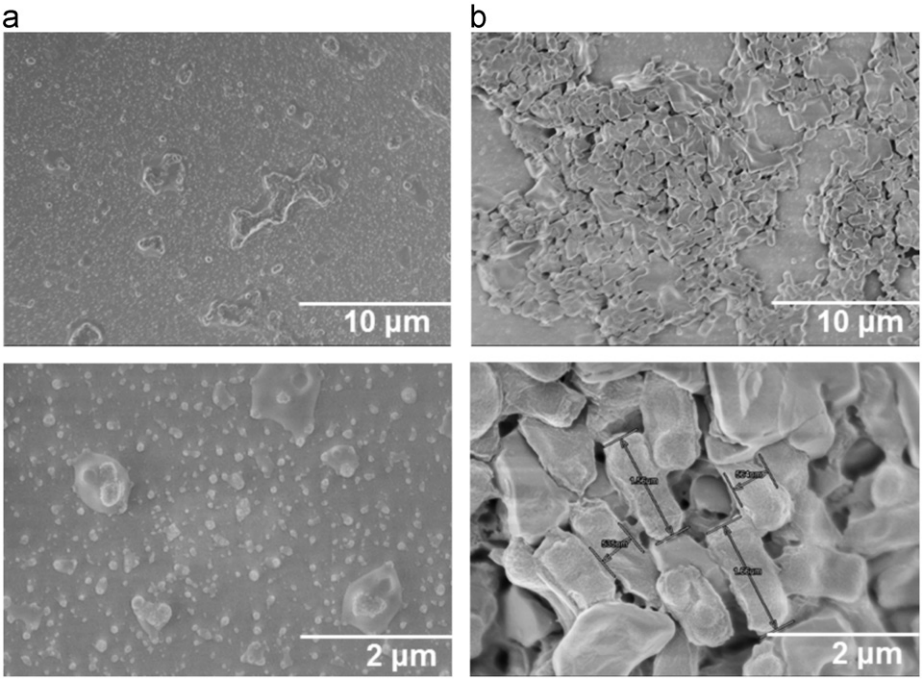

Fig. 3. SEM analyses for the morphological observation of (a) the MAP-coated surface and (b) immobilized cells on the MAP-coated surface. OPH-expressing *E. coli* cells (4 OD_{600}) were incubated on the MAP-coated surface at 37 °C for 1 h.

paraoxon (Sigma-Aldrich) solutions prepared in 100 mM 2-(*N*-cyclohexylamino)ethane-sulfonic acid (CHES) buffer (pH 9.0; Sigma-Aldrich) (Kang et al., 2005, 2006). Fifty-microliter aliquots of the paraoxon solutions were added to each microplate well containing MAP-immobilized recombinant *E. coli* expressing OPH. The hydrolysis reaction of paraoxon to *p*-nitrophenol (PNP) and diethylphosphoric acid was allowed to proceed for 5 min at room temperature. PNP was detected by measuring the absorbance at the λ_{max} of 410 nm (Kumar, D'Souza, 2010, 2011b) using a microplate spectrometer (Bio-Rad, Hercules, CA, USA). To investigate long-term storage stability, the whole cell array biosensor was tested at intervals of ~5 day using 1 mM paraoxon. The reusability of the whole cell array biosensor was also investigated using 100 μM paraoxon. To determine the applicability of the whole cell-based biosensor for the detection of an actual environmental sample, paraoxon-spiked samples with final concentrations ranging from 5 to 80 μM were prepared in tap water and applied to the biosensor.

3. Results and discussion

3.1. Construction of whole cell array biosensor using MAP as cell-immobilizing linker

Due to space limitations in the 96-well microplate, we first determined the optimal amounts of MAP coating and cell loading prior to construct the whole cell array biosensor. In a previous study, we demonstrated that MAP aggregation, which correlates with adhesion strength, was significantly influenced by pH and salt type (Lim et al., 2009). Varying quantities of MAP (50–500 μg/cm^2) were coated onto the well surface with a kosmotropic anionic ($HCOO^-$) and chaotropic cationic (Na^+) solution (pH 9.5), inducing both MAP aggregation with the proper size distribution and efficient adsorption (Lim et al., 2009). All tested MAP amounts showed similar cell-immobilizing efficiency at 1 and 10 OD_{600} (data not shown), indicating that the lowest quantity tested, 50 μg/cm^2 MAP, is sufficient to coat the well surface. We next investigated optimal cell loading in a range of 1–10 OD_{600} with a fixed quantity of MAP (50 μg/cm^2). We found that the degree of cell immobilization increased with increasing cell quantity, becoming saturated at approximately 4 OD_{600} (data not shown). Collectively, to form a whole cell array biosensor system based on MAP (Fig. 1), we determined that the optimal MAP coating was 50 μg/cm^2 and that the optimal cell loading was 4 OD_{600}.

We compared MAP-based cell immobilization with a simple physical adsorption-based method. We found that immobilized OPH-expressing *E. coli* cells on MAP-coated surface showed much higher paraoxon-degradative activity than simple adsorption-based immobilized cells (Fig. 2a), indicating that MAP facilitated the efficient immobilization of cells to the well surface. Fluorescence microscopy using DsRed-expressing recombinant *E. coli* also demonstrated that MAP was effective at immobilizing microbial cells. After repeated washings, there was almost disappearance of cells (~1359 ± 1229 cells/cm^2; ~0.36% of MAP-based cell immobilization efficiency) incubated on the bare surface, in contrast to the numerous cells (~375,854 ± 13,420 cells/cm^2) remaining adhered onto the MAP-coated surface (Fig. 2b). Note that for these experiments, prokaryotic cells were used. Previously, we showed that MAP is effective at immobilizing diverse types of eukaryotic cells (*Drosophila* S2, hamster CHO, mouse NIH/3T3, and human 293T) onto surfaces (Hwang et al., 2007a, 2007b, 2007c). This work, combined with our previous studies, demonstrated that MAP is an effective cell-immobilizing linker material for whole cell-based biosensors with multiple cell types.

Morphological observation of MAP-coated surfaces and immobilized cells on MAP-coated surfaces was performed using SEM.

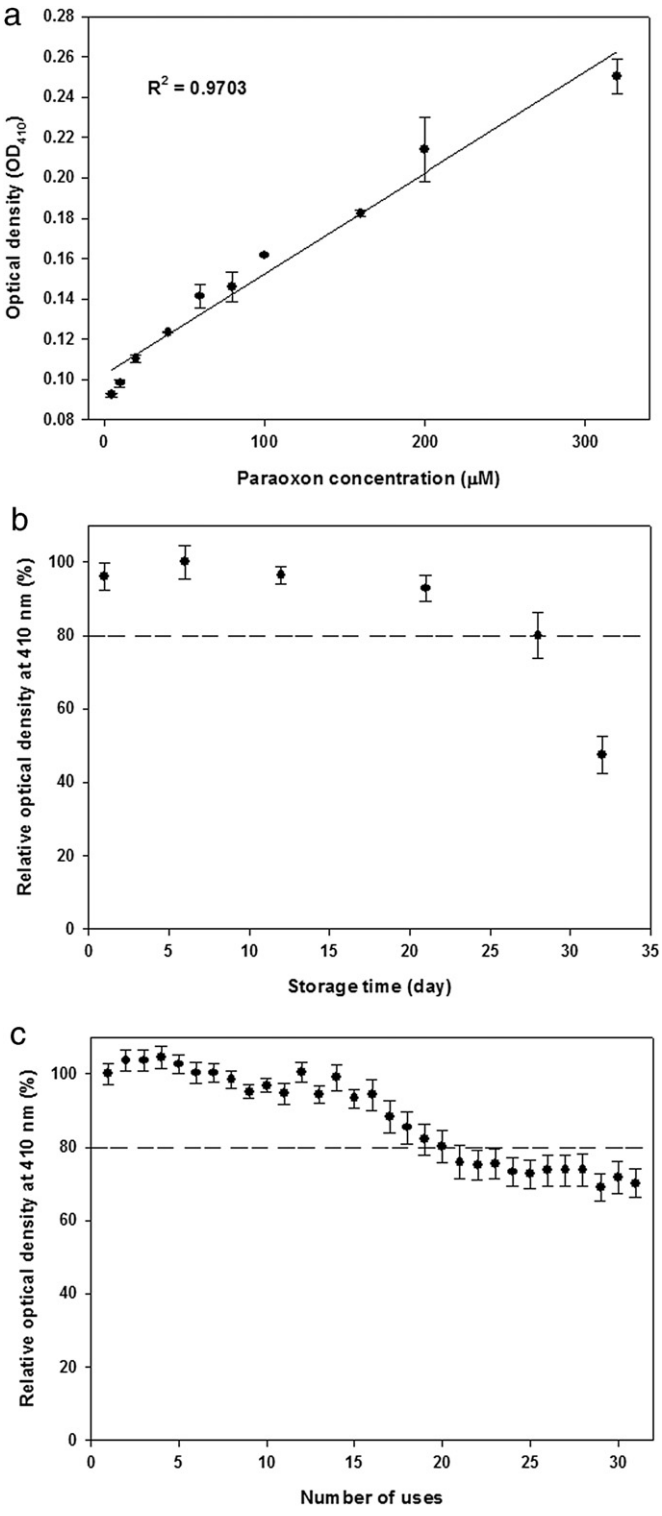

Fig. 4. (a) Quantitative detection of paraoxon using the whole cell array biosensor. The whole cell array biosensor, prepared with optimized conditions of MAP coating (50 μg/cm^2) and cell loading (4 OD_{600}), was incubated with various concentrations (5–320 μM) of paraoxon. (b) Long-term storage stability and (c) reusability of the whole cell array biosensor. The whole cell array biosensor was assessed at intervals of ~5 day using 1 mM paraoxon to examine its long-term storage stability. The reusability of the whole cell array biosensor was investigated by evaluating the repeated response to 100 μM paraoxon. The paraoxon hydrolysis reaction was performed at room temperature for 5 min and analyzed by measuring the absorbance at the λmax of 410 nm. Each value represents the mean of three independent wells (a) and eighteen independent wells ((b) and (c)), and the error bar is the standard deviation.

Consistent with our previous result (Lim et al., 2009), the coated surfaces showed numerous MAP aggregates (Fig. 3a) along with significant numbers of immobilized *E. coli* cells (size ~2 μm; Fig. 3b). These results indicate that MAP, which exists as an aggregate form, effectively immobilizes cells on coated surfaces without additional steps or pretreatment processes.

3.2. Quantitative detection, reproducibility, long-term stability, and reusability of the whole cell array biosensor.

A whole cell array biosensor was prepared by immobilizing recombinant *E. coli* expressing OPH in the periplasmic space on MAP-coated 96-well microplate. To investigate the feasibility of OP detection, the whole cell array biosensor was characterized by determining the quantitative detection, reproducibility, long-term storage stability, and reusability, which are all important issues in the development of a biosensor. First, we examined the quantitative detection by using different concentrations of paraoxon (5–320 μM). The absorbance intensity increased linearly with increasing paraoxon concentration with a high coefficient of determination (R^2) of over 0.97 in the tested range (Fig. 4a). Considering the signal intensity value (~0.05) at zero concentration of paraoxon and fixed reaction time (5 min), we determined that as little as 5 μM paraoxon could be detected with the whole cell array biosensor. This detection range is comparable to that of other whole cell biosensors using optical detection methods (Kumar et al., 2006; Kumar and D'Souza, 2010, 2011b; Mulchandani et al., 1998b). The reproducibility of each well on the whole cell array biosensor at 1 mM paraoxon was quite good (relative standard deviation (RSD)=3.70% for n=18). In addition, three distinct responses measured using same biosensor for 12 day showed a very low RSD of 1.91%. Thus, these results demonstrated a high reproducibility of this whole cell array biosensor for the detection of OPs. Next, the long-term storage stability of the whole cell array biosensor was evaluated by measuring the response of biosensor stored at 4 °C during the investigation period. We found that the whole cell array biosensor had excellent stability for 28 day with a retention of 80% activity (Fig. 4b), which is comparable to other whole cell biosensors (Kumar and D'Souza, 2011a, 2011b; Mulchandani et al., 1998b). However, the biosensor activity rapidly decreased to 47% of the original response by day 32. This decrease, which is similar to that described in other studies (Chen and Mulchandani, 1998; Mulchandani et al., 1998c), may be due to weakened transport machinery of OPH-expressing recombinant *E. coli* cells. Finally, the reusability of the whole cell array biosensor was investigated with 100 μM paraoxon. While the physically absorbed *E. coli* cells on the bare well surface were mostly washed away after a single use (data not shown), the whole cell array biosensor with recombinant *E. coli* cells immobilized on the MAP-coated well surfaces could be used for approximately 20 times with a retention of 80% activity (Fig. 4c). The strong adhesive property of MAP effectively prevented the immobilized cells from washing off of the sensor surface. From comparison of our MAP-based whole cell biosensor with other optical whole cell biosensor systems (Table 1), we found that the MAP-based system shows better long-term storage stability than glutaraldehyde crosslinking-based system (Kumar and D'Souza ,2010) and has generally fast response time compared to agarose entrapment-based system (Mulchandani et al., 1998b). Therefore, our novel whole cell array biosensor, formed using recombinant *E. coli* cells expressing OPH in the periplasmic space and immobilized onto MAP-coated microplate well surfaces, has excellent characteristics on reproducibility, long-term storage stability, response time, and reusability.

3.3. Detection of paraoxon in tap water using the whole cell array biosensor

Because OPs are potentially poisonous, practical and cost-effective analytical tools are needed to detect OPs in the environment (Lei et al., 2005; Kumar et al., 2006, Kumar and D'Souza, 2011b). We examined the feasibility of using a whole cell array biosensor to detect paraoxon in tap water. Samples of paraoxon dissolved in tap water were mixed with CHES buffer (pH 9.0) in a 3:2 ratio for optimal activity of OPH-expressing whole cells. The response of the whole cell array biosensor to paraoxon in tap water showed a very good linear correlation (R^2=0.9971), almost identical to that observed with paraoxon in distilled water, indicating that the additional components in tap water did not interfere with the ability of the whole cell array biosensor to

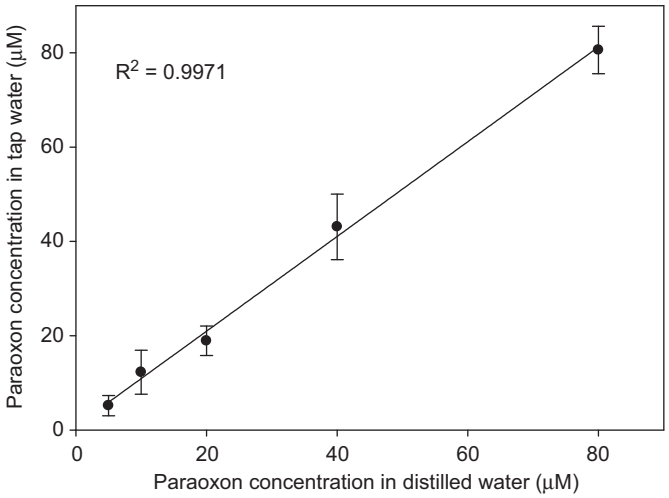

Fig. 5. Detection of paraoxon in tap water using the whole cell array biosensor. Tap water samples contained various concentrations (5–80 μM) of paraoxon. The paraoxon hydrolysis reaction was performed at room temperature for 5 min and analyzed by measuring the absorbance at the λ_{max} of 410 nm. Each value represents the mean of three independent wells, and the error bar is the standard deviation.

Table 1
Characteristic comparison of MAP-based whole cell biosensor with other optical whole cell biosensors for detection of Ops.

Immobilization method	Cell type	Detection range (μM)	Detection limit (μM)	Long-term storage stability	Response time (min)	Reference
Agarose-based physical entrapment	*E. coli* XL-1 blue cell	0–600	3 (paraoxon)	1 month	< 10	Mulchandani et al. (1998b)
Glass fiber filter paper-based physical entrapment	*Flavobacterium* sp. MTCC 2495	4–80	0.3 (methyl parathion)	1 month	< 3	Kumar et al. (2006)
Glutaraldehyde-based chemical crosslinking	*Sphingomonas* sp. JK1	4–80	4 (methyl parathion)	18 day	5	Kumar and D'Souza (2010)
Inner epidermis of onion bulb scale and glutaraldehyde-based crosslinking	*Sphingomonas* sp. JK1	4–80	4 (methyl parathion)	32 day	5	Kumar and, D'Souza (2011b)
MAP-based adhesion	*E. coli* BL21	5–320	5 (paraoxon)	28 day	5	This study

detect paraoxon (Fig. 5). These results indicate that our MAP-based whole cell array biosensor could be successfully used for detection of OPs in actual environmental samples.

4. Conclusion

In the present work, we investigated the use of MAP as an effective microbial cell-immobilizing linker for the development of a whole cell biosensor to detect neurotoxic OPs. Due to its strong adhesive property on diverse surfaces, MAP-based cell immobilization provided excellent efficiency and enhanced stability compared with a simple adsorption-based immobilization method. This whole cell array biosensor, which was prepared by the immobilization of periplasmic OPH-expressing recombinant *E. coli* cells on a MAP-coated surface in 96-well microplate, detected paraoxon at concentrations as low as 5 μM and demonstrated a quantitative detection range of ~5–320 μM with high reproducibility, good stability (80% activity remained for 28 day), and adequate reusability (up to 20 times). In simulating a real world challenge, the whole cell array biosensor was able to detect paraoxon in tap water without a reduction in sensitivity. Our results demonstrate that this whole cell array biosensor, constructed using MAP as a cell-immobilizing linker, can successfully detect neurotoxic OPs, indicating that this linker approach may be useful as a general platform for diverse whole cell-based biosensors.

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