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Short communication

Aqueous sol–gel encapsulation of genetically engineered *Moraxella* spp. cells for the detection of organophosphates

Diana Yu [a], Joanne Volponi [a], Swapnil Chhabra [a], C. Jeffrey Brinker [b], Ashok Mulchandani [c], Anup K. Singh [a,*]

[a] *Sandia National Laboratories, Livermore, CA 94550, USA*
[b] *Sandia National Laboratories, Albuquerque, NM 87185, USA*
[c] *Department of Chemical and Environmental Engineering, University of California, Riverside, CA 92521, USA*

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Abstract

An all-aqueous sol–gel method for encapsulation of bacterial cells in porous silicate matrices towards the development of a biosensor is described. The sol–gel encapsulation of cells is achieved at room temperature and neutral pH. Furthermore, use of sodium silicate as precursor avoids generation of alcohol that can be detrimental to cells in contrast to the traditional alkoxide sol–gel encapsulation process. *Moraxella* spp. cells engineered to express recombinant organophosphorus hydrolase (OPH) on the cell surface were encapsulated and OPH enzymatic activity was measured for paraoxon hydrolysis. Kinetic parameters (K_m and V_{max}) as well as pH behavior of surface-expressed OPH were determined to evaluate the effect of encapsulation. Cells encapsulated by the sodium silicate method displayed higher activity retention compared to those by the traditional alkoxide process. Time-course studies over a 2-month period indicate that immobilization through the sodium silicate process led to a reduction in activity of ∼5% as compared to ∼30% activity reduction in case of free cells in buffer indicating that immobilization leads to stabilization, a key parameter in biosensor development.

Keywords: Biosensors; Sol–gel; Organophosphates; Moraxella; Organophosphate hydrolase; Acetylcholine esterase

1. Introduction

Organophosphates are a class of toxic compounds that have found extensive use as pesticides and insecticides as well as potential chemical warfare agents (Singh et al., 1999). Exposure to high amounts causes inhibition of acetylcholinesterase leading to acetylcholine accumulation in synaptic junctions which may result in loss of muscle control, twitching, paralysis, unconsciousness, convulsions, coma, and ultimately death (Tuovinen et al., 1994). Organophosphorus hydrolases (aryldialkylphosphatase, EC 3.1.8.1) capable of hydrolyzing the phosphoric triester in organophosphorus nerve agents serve as prime candidates for the biological detection as well as detoxification of acetylcholinesterase inhibitors (Mulchandani et al., 1998; Tuovinen et al., 1994). In the present study, we evaluate the encapsulation of a recombinant organophosphorus hydrolase (OPH) expressed on the surface of *Moraxella* spp. cells.

Biosensors using immobilized receptors are finding ever-increasing applications in a wide variety of fields such as clinical diagnostics, environmental monitoring, food and drinking water safety, and illicit drug monitoring. Amongst numerous techniques being currently explored for the immobilization and integration of biological molecules, silica host matrices made by sol–gel process have emerged as a promising platform for encapsulation of enzymes, antibodies and cells (Al-Saraj et al., 1999; Bhatia et al., 2000; Coiffier et al., 2001; Fennouh et al., 1999, 2000; Gill, 2001; Lan et al., 1996; Livage et al., 2001; Yang and Saavedra, 1995). Silica matrices are relatively inexpensive to synthesize and have several desirable properties including chemical inertness, high mechanical strength, optical transparency, enhanced thermostability, biocompatibility, and resistance to microbial attack (Bhatia et al., 2000; Dunn

* Corresponding author. Tel.: +1 9252941260; fax: +1 9252943020.
E-mail address: aksingh@sandia.gov (A.K. Singh).

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et al., 1998; Lan et al., 2000). Traditional synthesis routes for silica matrices make use of tetramethyl orthosilicate (TMOS) or tetraethyl orthosilicate (TEOS) as the starting materials leading to the generation of methanol/ethanol as end products which are detrimental to the activities of encapsulated enzymes or cells (Coiffier et al., 2001). Recently, we reported an aqueous route for the synthesis of silica monoliths that uses sodium silicate as the precursor (Bhatia et al., 2000). This process can be carried out at a neutral pH without the generation of any alcohol and was first applied successfully for the encapsulation of glucose-6-phosphate dehydrogenase and horseradish peroxidase with 36–73% of native enzyme activity retention (Bhatia et al., 2000).

Typically, biosensors for organophosphate detection have been developed using immobilized enzymes which catalyze the hydrolysis of these compounds with the release of two protons per molecule hydrolyzed. Biosensing platforms exploiting immobilized OPH have been developed as fiber optic sensors (Mulchandani et al., 1999) and flow injection amperometric sensors (Mulchandani et al., 2001). The application of OPH in these sensing platforms involves the use of pure enzyme. Alternatively, the use of immobilized intact cells recombinantly expressing OPH on cell surfaces obviates the need for extensive purification steps that are involved in the use of free enzymes as well as lowers the possibility of enzyme leaching, thus simplifying the path for biosensor development. Biosensing platforms based on the use of intact *Escherichia coli* cells expressing OPH have been developed as potentiometric microbial electrodes as well as fiber optic sensors (Mulchandani et al., 1998).

In a recent study, *Moraxella* spp. cells were genetically engineered for surface expression of the pesticide hydrolyzing enzyme organophosphorous hydrolase (Shimazu et al., 2001). A truncated ice nucleation protein (INPNC) anchor was used to target the hydrolase onto the cell surface. The resulting recombinant strain was capable of targeting the INPNC-OPH fusion 70-fold more efficiently as compared to its *E. coli* counterpart (Mulchandani et al., 1998; Shimazu et al., 2001). Towards developing biosensing surfaces and matrices, we have explored the use of silica matrices as a platform for immobilization previously (Bhatia et al., 2000). Here, we describe the use of sodium silicate sol–gel process for encapsulating genetically engineered *Moraxella* spp. cells for the detection of organophosphate compounds. We compared this process with the traditional TMOS based sol–gel process and also examined the effects of pH and long term storage of the encapsulated OPH. Possible sensing platforms for organophospate detection using sol–gel encapsulated *Moraxella* spp. expressing OPH are also discussed.

2. Materials and methods

Sodium silicate was obtained from Fisher Scientific (Pittsburgh, PA). Dowex 50WX8-100 ion exchange resin, $CoCl_2$, and TMOS were purchased from Aldrich (Milwaukee, WI). Paraoxon was obtained from ChemService Inc. (West Chester, PA). Luria–Bertani (LB) medium and isopropylthio-β-galactoside (IPTG) were purchased from Invitrogen (Carlsbad, CA). Low-binding 96-well microtiter plates were obtained from Rainin Instruments (Woburn, MA). Deionized water at 18.2 MW (Milli-Q-Plus, Millipore, Ballerica, MA). Enzymatic activities were measured spectrophotometrically with SpectraMax 190 Microplate Spectrophotometer (Molecular Devices) equipped with a temperature controlled chamber.

2.1. Growth of Moraxella spp. cells

Moraxella spp. cells engineered for surface expression of OPH were constructed as described previously (Shimazu et al., 2001). A 50 ml LB culture containing 100 μg/ml kanamycin was innoculated with an overnight culture (3 ml) obtained from freezer stocks. Cells were grown at 30 °C with constant shaking at 300 rpm to an OD_{600} of 0.4 followed by induction with IPTG (1 mM final concentration) and additional growth for 48 h to ensure maximum surface expression.

2.2. Encapsulation of Moraxella spp. cells in silica sol–gel

Sol–gel solution was prepared as described previously (Bhatia et al., 2000) and stored at 4 °C. *Moraxella* spp. cells suspended in 1 M phosphate buffer were mixed with the sol–gel solution using a volumetric ratio of 1:5, and dispersed into 96-well plates, 50 μl per well. After 1 h, excess phosphate buffer (PB) was removed from each well and replaced with 100 μl of 20 mM CHES/50 μM $CoCl_2$ buffer at pH 9. The $CoCl_2$ was added to the buffer to enhance the specific activity of the enzyme. All plates were stored at 4 °C.

2.3. Encapsulation of Moraxella spp. cells in TMOS sol–gel

TMOS was mixed with deionized water in 1:2 molar ratio along with 220 μl of 0.02 N HCl. The mixture was sonicated for 15 min and refrigerated at 4 °C. As described for the silica sol–gel process, the cell solution was mixed with the sol–gel solution at a volumetric ratio of 2:3 and dispersed into 96-well plates, 75 μl per well. After 1 h, excess PB was removed from each well and replaced with 100 μl of 20 mM CHES/50 μM $CoCl_2$ buffer at pH 9. All plates were stored at 4 °C.

2.4. Kinetic assays for free and encapsulated Moraxella spp. cells

Optical density of the stock solution of *Moraxella* spp. cells in 1 M phosphate buffer was measured. Using a linear relationship between the cell concentration and its optical density, we extrapolated the absorbance of the reaction solution in each well and normalized the kinetics

data obtained from each well with the OD_{600} of the reaction solution. For free cell assays, the stock cell solution (10 μl) was added to paraoxon (140 μl) to obtain a final volume of 150 μl per well with paraoxon concentrations varying from 0.01 to 0.5 mM. For *Moraxella* spp. cells encapsulated in either the silica sol–gel or TMOS sol–gel, 100 μl of the appropriate paraoxon solution was added to each well to obtain a final volume of 150 μl per well with varying paraoxon concentrations from 0.01 to 0.5 mM. Rate of change of absorbance was measured at 405 nm using a spectrophotometer. Data collection at 12 s intervals for 30 min was allowed for each plate.

2.5. Effect of varying pH and long term storage

Paraoxon solutions at pH values varying from 5.5 to 10.0 were made using the corresponding phosphate buffers (100 μM PB/50 μM $CoCl_2$). Enzymatic activities were measured at each pH using a final substrate concentration of 0.5 mM paraoxon. For long term storage analysis, two plates of encapsulated cells were stored at 4 °C and samples from five wells were measured at regular intervals for at least 7 weeks using 5 wells per time point. Each well contained a final substrate concentration of 0.5 mM paraoxon. For comparison purposes, a suspension of *Moraxella* spp. cells in PBS (10 mM, pH 7.4) was prepared at the same cell concentration as encapsulated cells and activities measured at identical time points. All kinetics data collected were normalized to the initial reaction rate.

3. Results and discussion

Recombinant *Moraxella* spp. cells were successfully encapsulated in a sol–gel matrix via an aqueous route using sodium silicate as the precursor. As mentioned previously, traditional synthesis routes for sol–gel matrices use TMOS as the precursor. We compared this synthesis route for encapsulating *Moraxella* spp. cells with the sodium silicate method. Paraoxon was used as a model substrate to test the activity of the surface-expressed OPH encapsulated through both routes. Use of TMOS as the starting material could lead to generation of methanol/ethanol during the low temperature aging causing the encapsulated protein to denature over time. Consequently, TMOS encapsulated cells displayed lower activities as compared to those generated using sodium silicate when reacted with increasing paraoxon concentrations (Fig. 1). In addition, paraoxon hydrolysis by TMOS encapsulated OPH seemed to deviate from Michaelis–Menten kinetics, unlike that observed through the aqueous route. Note that the matrices produced through both routes differ slightly in their mean pore sizes (Bhatia et al., 2000). Leaching is an important consideration when comparing encapsulation of free enzymes as compared to surface-expressed enzymes on cell surfaces. The mean pore size for the silica sol–gel matrix is ∼200 Å

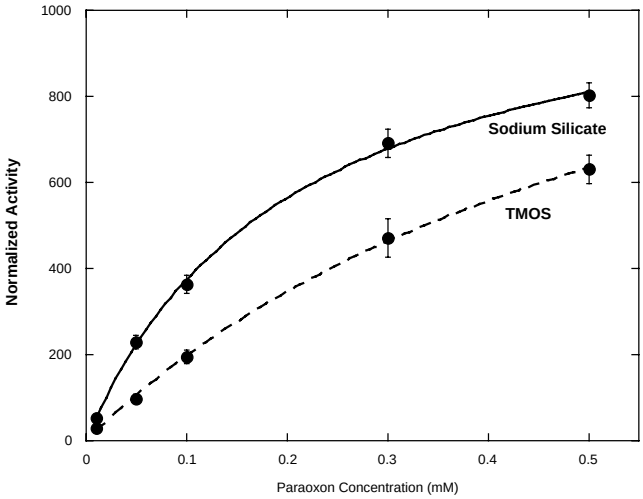

Fig. 1. Enzymatic activity of cell-surface-expressed OPH as a function of paraoxon concentration. Recombinant *Moraxella* spp. cells were encapsulated through the sodium silicate process and the tetramethyl orthosilicate (TMOS) process. Standard error for each data point is as shown.

(Bhatia et al., 2000) being sufficiently large to allow transport of small molecules such as substrates and products but exclude large particles like *Moraxella* spp. cells. Based on activity measurements of suspension solutions over several weeks, no leaching of *Moraxella* spp. cells expressing OPH was observed from the sol–gel matrix.

The sol–gel matrix maintained an adequate environment for expressed OPH to maintain native conformation and original enzymatic activity. Hydrolysis of paraoxon by encapsulated cells followed typical Michaelis–Menten kinetics ($V = V_{max}S/[K_m + S]$), where V is the initial reaction rate, S is the substrate concentration; K_m and V_{max} are Michaelis–Menten constants corresponding to the binding constant and maximum reaction velocity, respectively) as also observed for free cells (Fig. 2). The estimated Michaelis–Menten parameters for surface-expressed OPH in free ($R^2 = 0.9972$) and encapsulated cells ($R^2 = 0.9990$) were as follows: free cells: K_m, 0.16 mM; V_{max}, 2221 AU/min OD_{600} and encapsulated cells: K_m, 0.58 mM; V_{max}, 1232 AU/min OD_{600}. Maximum activity for paraoxon hydrolysis by OPH expressed in encapsulated cells was ∼55.5% of that of the freely suspended cells whereas the ratio of corresponding binding constants in encapsulated versus freely suspended cells was ∼3.6. Since V_{max} is a function of the enzyme conformation, a decrease in maximum activity upon encapsulation would indicate that the immobilization process affects OPH conformation to some extent. In addition to active site conformation, the binding constant, K_m also depends on substrate accessibility to the active site. An increased binding constant in the encapsulated enzyme would, therefore, indicate less specific binding perhaps due to a changed conformation in addition to restricted substrate accessibility in encapsulated cells.

An important consideration in the development of enzyme-based biosensors lies in identifying conditions for most sensitive operation of the immobilized enzyme that

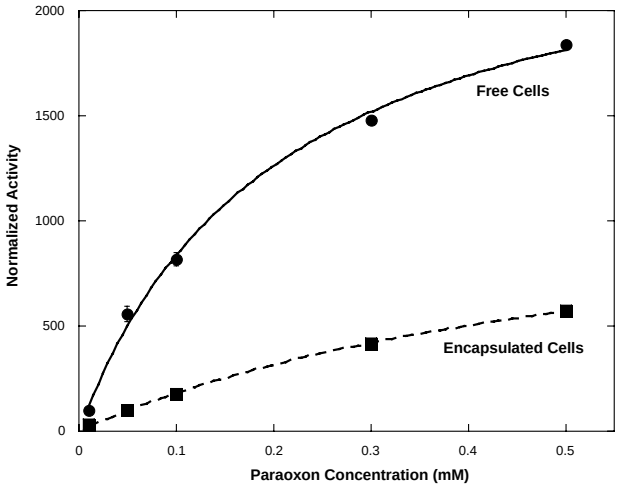

Fig. 2. Michaelis–Menten kinetics of paraoxon hydrolysis using cell-surface-expressed OPH. Comparison of free and encapsulated (sodium silicate process) recombinant *Moraxella* spp. cells. Free cells: K_m, 0.16 mM; V_{max}, 2221 AU/min OD_{600}; encapsulated cells: K_m, 0.58 mM; V_{max}, 1232 AU/min OD_{600}. Standard error for each data point is as shown.

may differ from those known for the native enzyme. To determine conditions for optimum detection, we tested activities of free and encapsulated OPH over a wide range of pH values. As shown in Fig. 3, encapsulated OPH displayed lower sensitivities under acidic conditions. For pH values between 6 and 8, the sensitivity increased with highest activities being observed under highly basic conditions. The increase in rate of paraoxon hydrolysis with pH to a limiting value at high pH indicates titration of a single base in the enzyme active site (Whitaker, 1972). Note that freely suspended and encapsulated cells displayed similar activity profiles with the activity versus pH curves shifted to higher pH values for the immobilized enzyme. This may be due to the fact that under neutral conditions, silanol groups in a silica matrix are negatively charged and the electrical double layer consists of primarily cationic buffer ions and hydrogen ions, thereby, reducing the pH of the double layer below that of the bulk solution. The immobilized enzyme within this double layer is, therefore, exposed to a pH lower than that in the bulk solution. Thus, immobilization of OPH in the sol–gel matrix alters the microenvironment in which the enzyme operates thereby causing a shift in the pH profile.

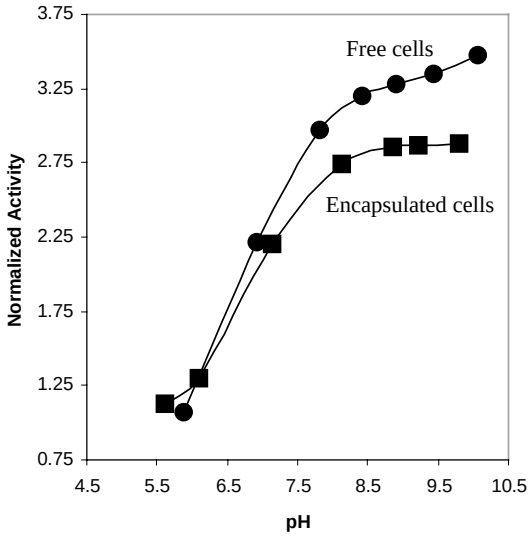

Fig. 3. Enzymatic activity of cell-surface-expressed OPH as a function of solution pH. Comparison of free and encapsulated (sodium silicate process) recombinant *Moraxella* spp. cells.

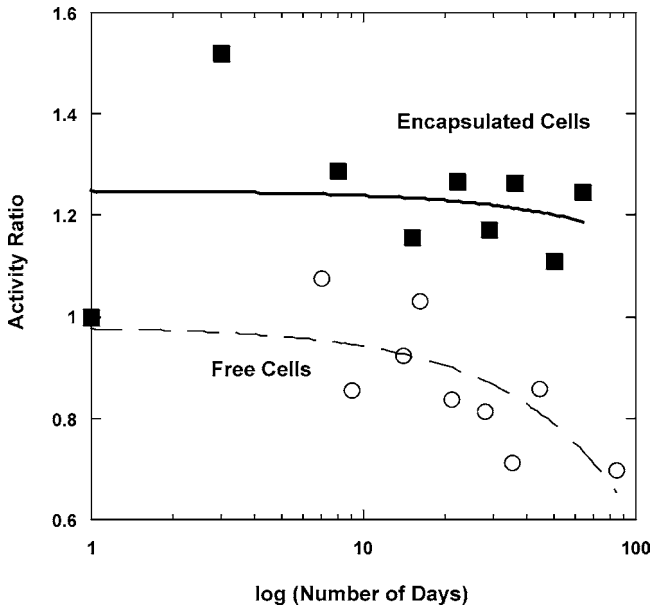

Fig. 4. Effect of long term storage on the activity of cell-surface-expressed OPH in free and encapsulated *Moraxella* spp. cells. Although encapsulation leads to lowered OPH activity as compared to free cells, encapsulated cells maintain OPH activity over a longer period as compared to free cells.

Encapsulation of OPH not only affords ease of handling and usage but also prolongs shelf life of the enzyme. We performed a time study to evaluate the effect of encapsulation on OPH activity. As shown in Fig. 4, a slight increase in activity through day 3 was followed by a steady decrease through the remaining time period. Loss in functionality of the freely suspended OPH occurred at ∼4 times that of the encapsulated enzyme. Encapsulated OPH would be effective in its detection of paraoxon for up to 64 days retaining ∼95% of its original activity. Although encapsulation leads to lowering of activity of the surface-expressed OPH as compared to that in free cells, the lowered activity in encapsulated cells is maintained over an extended period of time. Thus, encapsulation of OPH in the sol–gel matrix imparts enhanced stability also an important consideration in biosensor development.

Another concern that cell-based biosensors face is that whether cells are live or viable upon encapsulation. For the current application, it is not essential that cells be alive, but it is crucial that they maintain their shape and membrane-integrity to avoid losing the membrane-anchored OPH. A compromised membrane can also result in decrease in activity of OPH. The membrane-integrity was assessed using Live/Dead BacLight Bacterial Viability assays

(Molecular Probes, Eugene, OR) which utilize a mixture of SYTO 9 green-fluorescent nucleic acid stain which labels cells with both intact and damaged membranes, and a red-fluorescent nucleic acid stain, propidium iodide, which penetrates only cells with compromised membranes. This assay indicated that cells used in this study had intact cell membranes.

4. Conclusion

Cell-based sensors are useful in many applications because of the advantages they offer—they eliminate the need to purify proteins derived from these cells and also present receptor proteins or sensing enzymes in high copy numbers in their native environment. The key challenge associated with cell-based biosensing is to use an immobilization or encapsulation process that keeps cells intact and active. The all-aqueous sol–gel process using physiological pH and ionic strength proposed here is a generic encapsulation technique that can be applied to many different cell types and can also be extended to encapsulation of organelles and tissues. The sol–gel process allows formation of sensor matrices in many forms—they can be cast as bulk particles, can be spin coated on a flat sensor surface (e.g., fiber optic), or cast inside a capillary or tube to develop flow-through sensors.

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