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Development of sensors for direct detection of organophosphates. Part I: immobilization, characterization and stabilization of acetylcholinesterase and organophosphate hydrolase on silica supports

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Abstract

Biosensors for organophosphates in solution may be constructed by monitoring the activity of acetylcholinesterase (AChE) or organophosphate hydrolase (OPH) immobilized to a variety of microsensor platforms. The area available for enzyme immobilization is small (<1 mm^2) for microsensors. In order to construct microsensors with increased surface area for enzyme immobilization, we used a sol–gel process to create highly porous and stable silica matrices. Surface porosity of sol–gel coated surfaces was characterized using scanning electron microscopy; pore structure was found to be very similar to that of commercially available porous silica supports. Based upon this analysis, porous and non-porous silica beads were used as model substrates of sol–gel coated and uncoated sensor surfaces. Two different covalent chemistries were used to immobilize AChE and OPH to these porous and non-porous silica beads. The first chemistry used amine-silanization of silica followed by enzyme attachment using the homobifunctional linker glutaraldehyde. The second chemistry used sulfhydryl-silanization followed by enzyme attachment using the heterobifunctional linker N-γ-maleimidobutyryloxy succinimide ester (GMBS). Surfaces were characterized in terms of total enzyme immobilized, total and specific enzyme activity, and long term stability of enzyme activity. Amine derivitization followed by glutaraldehyde linking yielded supports with greater amounts of immobilized enzyme and activity. Use of porous supports not only yielded greater amounts of immobilized enzyme and activity, but also significantly improved long term stability of enzyme activity. Enzyme was also immobilized to sol–gel coated glass slides. The mass of immobilized enzyme increased linearly with thickness of coating. However, immobilized enzyme activity saturated at a porous silica thickness of approximately 800 nm. Published by Elsevier Science S.A.

Keywords: Organophosphate; Sensor; Organophosphate hydrolase; Acetylcholinesterase; Enzyme; Immobilization; Sol–gel; FET; ISFET; pHFET; EnFET; AChE; OPH

1. Introduction

There is an enduring need for improved sensors or techniques to detect organophosphates in the air, soil, and water as well as in food. Organophosphates are extremely potent inhibitors of the enzyme acetylcholinesterase (AChE) found in cholinergic neurons. If AChE activity is blocked, acetylcholine accumulates at cholinergic receptor sites thereby excessively stimulating the cholinergic receptors. This can lead to various clinical complications including fibrillation, leading ultimately to death. In addition, long term exposure to low levels of organophosphates can produce persistent and additive inhibition of acetylcholinesterase resulting in a delayed neuropathy (Williams et al., 1997). Organophosphates have been used extensively as toxic agents, in the form of agricultural insecticides such as parathion, fenthion, dimpylate and malathion; and chemical warfare agents, commonly known as 'nerve gases', such as sarin, soman and tabun.

The majority of biosensors for organophosphates to date have utilized the enzyme AChE as the recognition

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component. Presence of organophosphate blocks the enzyme activity thereby leading to decreased device response. Hence, in an AChE-based biosensor, signal is inversely proportional to organophosphate concentration. In the last few years, another enzyme, generically termed organophosphate hydrolase (OPH), has been proposed by researchers as an alternate recognition component (Dumas et al., 1990; Dave et al., 1993; Rainina et al., 1996). This enzyme hydrolyzes an organophosphate molecule, leading to products that can be monitored spectrophotometrically or electrochemically. Because the organophosphate is the substrate for OPH, this scheme leads to a direct determination of analyte as the rate of signal generation is directly proportional to the concentration of organophosphate. In this paper, we investigated two covalent immobilization chemistries with both AChE and OPH, as well as a novel sol–gel surface modification to enable development of enzyme modified field effect transistors (FET) for detection of organophosphates. Sensor response is based upon detection of pH changes resulting from hydrolysis of acetylcholine or organophosphate compounds by immobilized enzyme.

With few exceptions, the factor that limits the performance of a biosensor is the biological component. Development of an efficient biosensor requires schemes for immobilizing adequate amount of the recognition element (e.g. enzyme) while maintaining its biological activity. To avoid thick membrane strategies, which may pose analyte diffusion barriers and limit sensor response (Williams and Blanch, 1994), we selected covalent attachment for immobilizing enzyme directly to the gate insulator of a pH-sensitive FET. One of the essential factors in choosing an immobilization scheme is reproducibility; the method must immobilize biological molecules with minimal sensor-to-sensor variation with respect to mass and activity of immobilized molecules. Two different silanization chemistries followed by two different routes of protein immobilization were investigated. The first immobilization was based upon aminopropyltriethoxysilane (APTS) derivitization followed by glutaraldehyde (Weetall, 1969; Vandenberg et al., 1991; Weetall, 1993; Flounders et al., 1995) the second used a mercaptopropyltrimethoxysilane (MPTS) derivitization followed by a heterobifunctional

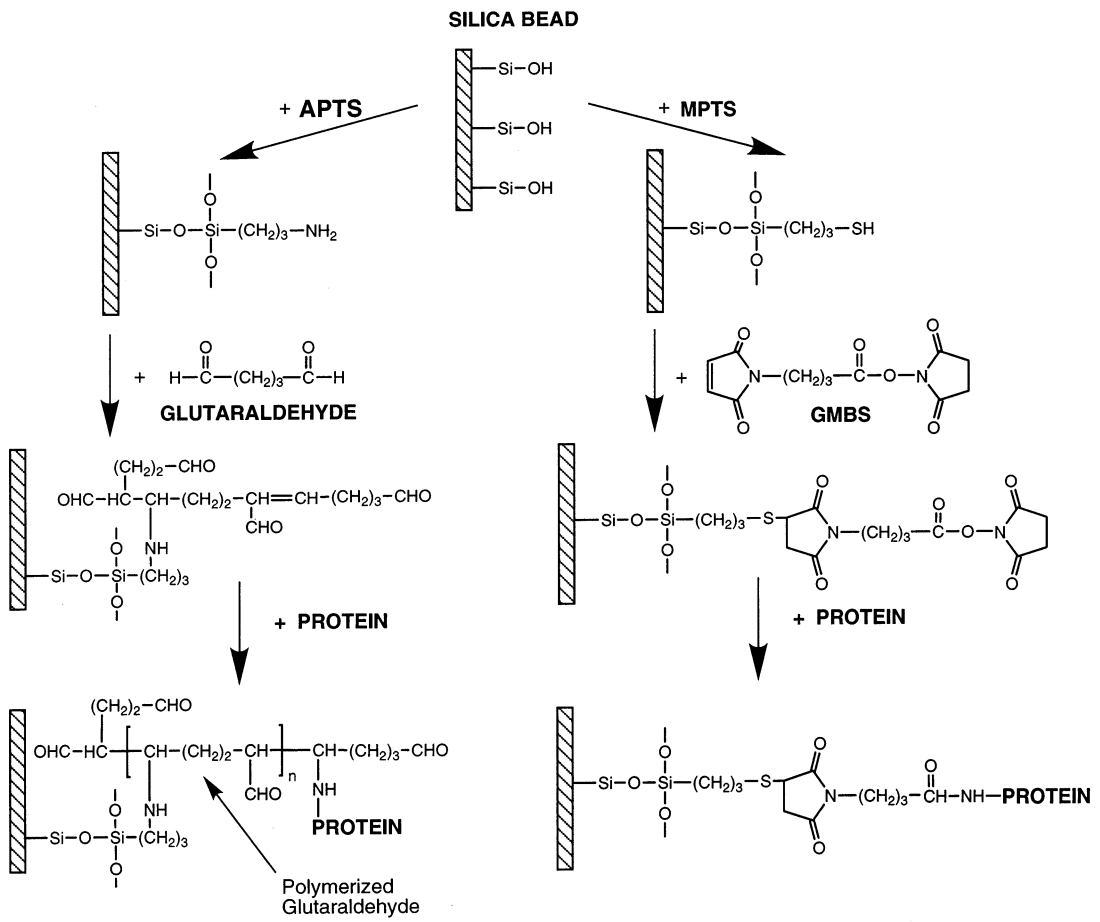

Fig. 1. Comparison of the two different chemistries used for covalent immobilization of proteins to glass slides dip-coated with silica microspheres and to porous and non-porous silica beads. APTS, 3-aminopropyltrimethoxysilane; MPTS, mercaptopropyltrimethoxysilane; GMBS, *N*-γ-maleimidobutyryloxy succinimide ester.

crosslinker (*N*-γ-maleimidobutyryloxy succinimide ester, GMBS) (Bhatia et al. 1989, 1991, Fig. 1). To minimize non-specific protein adsorption, the effect of inclusion of a non-ionic surfactant, Tween-20, in the reaction mixture was also investigated. Stability of the enzyme coatings is also critical, and was monitored by measuring activity of immobilized enzyme for approximately 12 months.

Although APTS/glutaraldehyde is a well-established route for conjugating biomolecules to silica surfaces, its use raises specific reproducibility concerns. First, in an aqueous environment, all three ethoxy groups of APTS hydrolyze and are available for conjugation to silanol groups present on silica surface or other silane molecules. This may lead to formation of silane polymers on the silica surface. Second, it has been reported that glutaraldehyde polymerizes with other glutaraldehyde molecules forming an ill-defined, multilayered structure. Formation of polymeric structures, either during APTS silanization or glutaraldehyde treatment, can result in loss of immobilization reproducibility. The second immobilization scheme was defined to specifically avoid these polymerization issues. MPTS was deposited from dry toluene to control methoxy hydrolysis and a heterobifunctional crosslinker (GMBS) was used to eliminate cross linker self-condensation. In principle, the later scheme should result in monolayer protein attachment and enhanced reproducibility.

A pH-sensitive FET has a very small gate area available for enzyme immobilization. This, in turn, limits the amount of biocomponent that can be immobilized. One way to increase the surface area available for immobilization for a given sensor dimension is to use a sol–gel process to create a porous silica structure (Dave et al., 1994) on the sensor surface. In a typical enzyme modified FET, the exposed surface layer is SiO_2 (oxidized silicon nitride) on top of a stacked Si_3N_4/SiO_2 gate insulator. The sol–gel process involves hydrolysis of alkoxide precursors under acidic or basic conditions, followed by polycondensation of the hydroxylated units to from a porous gel. The gel consists of Si–O–Si bonds, similar to the exposed gate SiO_2 layer, and hence there is no change expected in terms of electrical and structural properties.

A number of researchers have investigated acid catalyzed sol–gel formation with the goal of entrapping biomolecules during sol–gel formation (Wang et al., 1993; Dave et al., 1994; Lan et al., 1996). Although this leads to a very large number of incorporated molecules, formation of alcohol as a byproduct tends to denature the entrapped protein. Presence of labile biomolecules restricts the temperature of reaction to 4–40°C, and often the resulting sol–gel structure is not structurally stable. A significant portion of entrapped macromolecules may also be inaccessible to their ligands and substrates. An alternate approach, the one we have explored, is use of a sol–gel process to form a structurally stable porous matrix followed by biomolecule immobilization. Uniform silica microspheres were formed via a base catalyzed sol–gel process. These silica microspheres, often referred to as Stöber or SFB spheres (Stöber et al., 1968; Brinker and Scherer, 1990), have a very uniform size distribution; typically $< 5\%$ of the particles differ by more than 8% from the mean size. A porous matrix was then formed by depositing SFB microspheres via dip-coating on planar surfaces and curing at 300–400°C. High temperature treatment drives the reaction to completion and also volatalizes alcohol produced during reaction, thereby producing a clean, stable matrix. Enzyme was then attached using one of the selected immobilization chemistries. This not only avoids the exposure of proteins to incompatible conditions used during sol–gel formation, but also circumvents the inaccessibility issue.

The sol–gel process used is very similar to the one used for manufacturing porous silica particles; hence it is not surprising that a sol–gel coated sensor surface resembles the surface of porous silica. Scanning electron microscopy confirmed that the two surfaces have similar size, shape and density of pores. Taking advantage of this resemblance, porous and non-porous silica particles were used as model immobilization platforms to represent sol–gel coated and uncoated FET surfaces for determination of optimal immobilization conditions. Enzyme was attached covalently to the porous and non-porous silica particles using one or the other of the above described chemistries. Experimentally, it is expensive and cumbersome to use FETs for these studies. Silica particles are much easier to use, much less expensive, and readily available in a variety of pore sizes.

Our results showed that both immobilization strategies were effective for immobilization of the two enzymes. Use of Tween-20 during the enzyme immobilization was key to minimizing non-specific adsorption of enzyme to silica. The total activity was much greater for immobilization to porous substrates, as expected as a result of their much greater surface area. More importantly, the long term stability of enzyme immobilized to porous substrates was much greater than that of enzyme immobilized to non-porous substrates.

2. Methods and materials

Materials: Porous silica beads (Nucleosil 1000-7, Alltech, Deerfield, IL) and non-porous silica beads (Bangs Laboratories, Inc., Fishers, IN), were used as immobilization supports. AChE was from Electrophorus electricus (Boehringer Mannheim Corp., Indianapolis, IN). OPH was generously provided by Dr. James

Wild of Texas A and M University. OPH isolation and purification have been previously described (Lai et al., 1994). The silane agents used were 3-aminopropyl triethoxysilane (APTS) and 3-mercaptopropyl trimethoxysilane (MPTS), (Sigma, St Louis, MO). The crosslinkers were glutaraldehyde (Grade 1, Sigma, St. Louis, MO) and GMBS (Pierce, Rockford, IL). Assays were performed using bicinchoninic acid (BCA) protein assay kit (Pierce), propionylthiocholine (PTC) enzyme activity assay kit (Sigma), and paraoxon (Chem Services, Inc., West Chester, PA). Phosphate buffered saline (PBS, Sigma), pH 7.4 contained 10mM sodium phosphate, 138 mM NaCl, 2.7 mM KCl. For sol–gel coatings tetraethoxysilane (TEOS), (Aldrich Chemicals, Milwaukee, WI) was used. All other chemicals were from Sigma or Aldrich and of reagent grade or better.

2.1. Sol–gel sphere formation and coating

Tetraethoxysilane was hydrolyzed in a solution of ammonium hydroxide (70%) in water and absolute ethanol to give a solution pH of 11.6. Silica microspheres (SFB spheres) begin to form quickly upon vigorous stirring at room temperature. Upon completion of reaction, particle suspension can be stored for months. Coatings were deposited by dip-coating glass slides into the particle suspension using a microprocessor-controlled linear translation stage and microstepping motor. The coating operations were performed in an inert atmosphere glove-box to control drying conditions (relative humidity $< 5\%$). Prior to coating, slides were cleaned in an oxygen plasma chamber (500 W, 1 torr, 30 min) to remove organic contamination. Coatings were deposited at a rate of 8 inches per minute, followed by firing in an upright position at 400°C for 10 min in air. When multiple coatings were deposited, firing steps were performed between each coating.

2.2. Cleaning and activation

All reactions with silica beads were performed with vigorous shaking or rotation to keep particles suspended; reactions with sol–gel coated slides were performed in a custom made Teflon® reaction vessel. Silica beads weighing 300 mg were washed with 5 ml of methanol in a sonication bath to remove any organic residue. After rinsing, 5 ml of water was added and beads were heated in a boiling water bath for 15 min. After decanting off the excess water, 10% (2.9 M) hydrogen peroxide was added and the solution was heated for 1 h in a boiling water bath. Silica particles then were rinsed with water and methanol and dried at 90°C overnight.

Sol–gel coated slides were washed sequentially with methanol (room temperature, 5 min, with sonication), deionized water (90°C, 15 min), 10% hydrogen peroxide (90°C, 1 h) and then dried at 90°C overnight.

2.3. APTS/glutaraldehyde treatment

Silica beads or sol–gel coated slides were soaked in 10% (v/v) in deionized water (80°C, 3 h); pH was adjusted to 7 with glacial acetic acid. After rinsing, samples were soaked in 10% (v/v) glutaraldehyde in deionized water (room temperature, 1 h). Samples were rinsed extensively after glutaraldehyde treatment to remove any adsorbed crosslinker.

2.4. MPTS/GMBS treatment

Silica beads or sol–gel coated slides were soaked in anhydrous toluene (room temperature, 5 min, under vacuum). Samples were then transferred to a glove-box that had been flushed with dry N_2. The relative humidity inside the glove-box was maintained at approximately 25%. MPTS was added to the reaction mixture to make a 5% (v/v) solution of silane. The reaction was carried out for 2 h at room temperature. Samples were then washed sequentially with toluene and ethanol. Samples were then soaked in GMBS (8 mM in ethanol, room temperature, 1 h). Samples were rinsed sequentially with ethanol, water and PBS.

2.5. Protein attachment

Silica beads or sol–gel coated slides were soaked in 0.1–0.2 mg/m AChE or OPH in PBS with 0.02% NaN_3 (4°C, overnight). Results were compared with and without 0.05% (w/v) Tween-20 added to the reaction mixture to minimize protein adsorption. Samples were rinsed with PBS then incubated in 100 mM glycine for 30 min to block any unreacted aldehyde or succinimidyl esters. Samples were then soaked with intermittent vigorous shaking (room temperature, 30 min) sequentially in 0.05% Tween-20 and 0.5 M NaCl in PBS to remove non-specifically adsorbed protein. Samples were then stored in PBS with NaN_3 at 4°C for later use.

2.6. Protein assay

Protein assays were performed using the BCA assay. Unreacted porous and non-porous bead samples, sol–gel coated slides, as well as PBS buffer alone were used as controls. Standards, samples and controls were added to glass test tubes in duplicates along with working reagent in a 1:1 ratio and heated in a 60°C oven for 60–90 min. Vials were vortexed to achieve good mixing every 10 to 15 min during the heating process. Samples containing beads were centrifuged and supernatant was collected and absorbance at 562 nm was read on a microplate reader (Bio-Tek Instruments EL 340).

2.7. AChE and OPH activity assay

Bead samples were vortexed to equally distribute the beads in solution. Glass slides with and without sol–gel coats (1.8 × by 1.8 cm squares) were broken into pieces that would fit into cuvettes. AChE enzyme activity was measured using Cholinesterase (PTC) Reagent Kit. The absorbance of solution was monitored at 405 nm for 1–2 min using a Varian CARY 3 UV-Vis Spectrophotometer. Kinetic parameters for free and immobilized AChE were determined using Michaelis–Menten kinetics corrected for substrate inhibition (Radic et al., 1990), given by:

$$V = \frac{V_{max}}{1 + K_1/S + S/K_2} \quad (1)$$

where V is the reaction rate, S is the substrate concentration, V_{max} is the maximum reaction rate, K_1 is equilibrium constant for enzyme–substrate complex, and K_2 is equilibrium constant for the substrate inhibition step.

OPH enzyme activity was measured using paraoxon as substrate. OPH hydrolyzes paraoxon to diethyl phosphate and *p*-nitrophenol which has an absorption maxima at 405 nm. Paraoxon stock solution was made at 10 mM and was diluted to 2 mM in 20 mM 2-[*N*-cyclohexylamino]-ethanesulfonic acid (CHES), pH 9.0 just before use. Broken glass slides or 10–40 μl of beads were added to a cuvette containing 1.35 ml of 20 mM CHES. Paraoxon (150 μl, 2 mM) was added and the reagents were mixed by repeated pipetting. Absorbance was monitored at 405 nm for 1–2 min. Kinetic parameters for free and immobilized OPH were determined using the non-modified Michaelis–Menten equation, given by:

$$V = \frac{V_{max} S}{K_m + S} \quad (2)$$

where V is the reaction rate, S is the substrate concentration, V_{max} is the maximum reaction rate, and K_m is the apparent Michaelis constant.

In Eqs. (1) and (2), the maximum reaction rate V_{max} can also be written as $V_{max} = k_{cat} E_0$ where k_{cat} is the catalytic constant of enzyme and E_0 is the total enzyme concentration. Throughout this report, activity is presented as 'total activity' (V_{max} multiplied by a constant, having units of absorbance at 405 nm per minute per mg of beads) or 'specific activity' (k_{cat} multiplied by a constant, having units of absorbance at 405 nm per minute per μg of enzyme). Specific activity was calculated by dividing total activity by amount of immobilized enzyme as determined by the BCA assay.

2.8. Stability of enzyme (OPH and AChE) on porous and non-porous beads

The activity of enzyme, OPH or AChE, immobilized on porous and non-porous silica beads was measured periodically as described above. AChE bead samples were stored in PBS with sodium azide (0.02%) and kept at 4°C. OPH bead samples were stored in PBS only. Bead samples were allowed to reach room temperature and were vortexed prior to activity assays.

3. Results and discussion

3.1. Particle characterization

Fig. 2 shows scanning electron micrographs of glass surfaces that have been coated with monodisperse spheres (200 nm) using a sol–gel process. One sol–gel coating results in a partial monolayer of sol–gel spheres on the glass surface. A second coat of sol–gel provides nearly a complete monolayer and at some places a second layer can be seen as well. After a third coat, the original glass surface is hardly visible. The sideview of a glass slide with eight coats of sol–gel clearly demonstrates the porous structure; with multiple coatings the matrix of packed spheres mimics the surface of a porous silica particle. The reason for this similarity stems from the fact that very similar processes are used to make sol–gel coatings and porous silica particles. In both cases, silica nanospheres are crosslinked using an orthosilicate (e.g. tetraethoxysilane, TEOS or tetramethoxysilane, TMOS) to form highly porous structures. Fig. 3 compares scanning electron micrographs of a 7 μm silica particle with 100 nm pores and a glass slide coated with eight layers of 200 nm diameter SFB microspheres. Although there are some structural differences because of different sizes of starting nanospheres, the porosity of a single 7 μm bead is very similar to the porosity of the sol–gel coated surface. In this paper we have employed commercially available porous and non-porous silica beads to represent sol–gel coated and uncoated surfaces, respectively.

An important factor in judging the effectiveness of an immobilization method is to measure the amount of protein immobilized to a sample as well as the total activity of that sample so that specific activity (enzyme activity per amount of enzyme) can be determined. It was the need to perform these multiple measurements (and perform them multiple times) with equivalent samples that led to the use of silica beads as model immobilization substrates. Preparation of the vast number of sol–gel coated planar surfaces needed for such experiments was not reasonable.

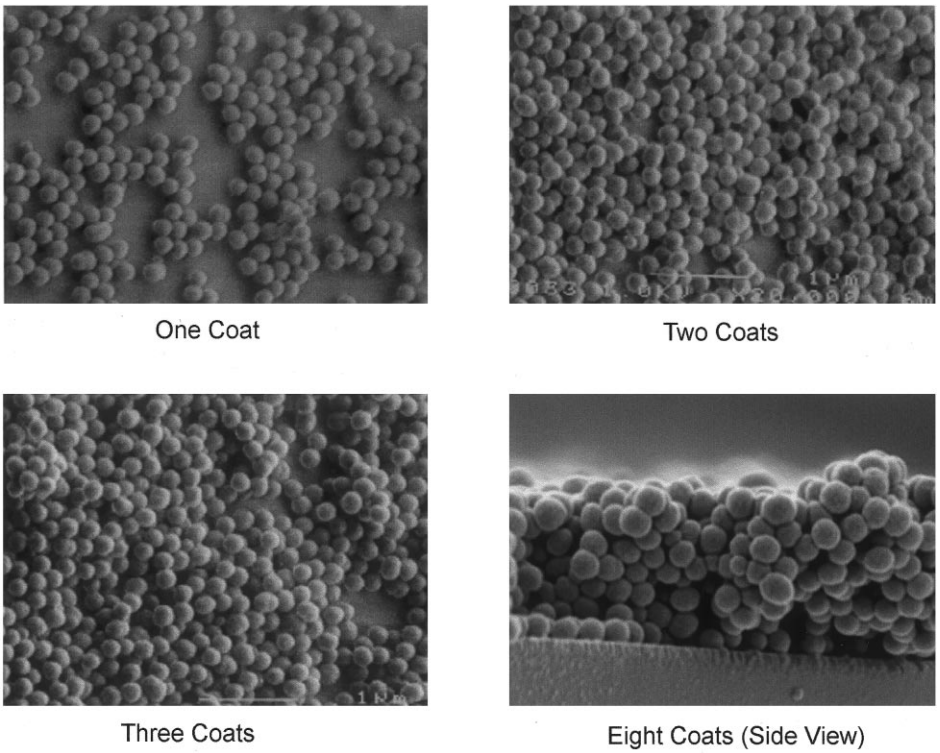

Fig. 2. Scanning electron micrographs of glass slide dip-coated with 200 nm silica microspheres. Silica microspheres were formed via a base catalyzed sol–gel process. Samples were fired at 400°C in air for 10 min after each coat was applied.

AChE and OPH were immobilized to porous and non-porous silica beads using both immobilization chemistries, and activity and enzyme load were determined for all cases. From these measurements, comparisons of total activity and specific activity for all cases was possible.

3.2. Immobilization of acetylcholinesterase

Fig. 4 compares the effectiveness of the two immobilization chemistries in terms of amount of AChE immobilized and total AChE activity for porous and non-porous silica particles. As a result of the much greater surface area of the porous particles, there is significantly greater enzyme loading and enzyme activity for porous particles compared with non-porous particles. In the case of porous beads (Fig. 4a), the enzyme load was significantly less with the GMBS than with the glutaraldehyde chemistry while the total activity was approximately the same with both chemistries. In other words, the GMBS chemistry attached less enzyme but enzyme was more active (greater specific activity) than enzyme immobilized with glutaraldehyde. For non-porous silica (Fig. 4b), glutaraldehyde linkage performed better than GMBS both in terms of enzyme load and total activity.

Fig. 4 also shows the extent of non-specific adsorption of enzyme (labeled 'No Chemistry' in the figure) to silica supports. In both type of support, enzyme activity from non-specifically adsorbed enzyme is minimal (less than 5% of the enzyme activity of covalently bound enzyme). For non-porous support, there appears to be a significant amount of non-specific adsorption ($\sim 25\%$ of enzyme activity of covalently bound protein) but the adsorbed enzyme has virtually no activity. Silica surfaces are notorious for physical adsorption of proteins, the predominant causes of adsorption being electrostatic and hydrophobic interaction (Duncan et al., 1995). In this study, a non-ionic surfactant (Tween-20) was used in the reaction mixture as well as in the post-reaction washes to reduce hydrophobic interaction. Inclusion of Tween-20 in post-reaction wash resulted in a small reduction in non-specific adsorption but its addition to the reaction mixture enabled reduc-

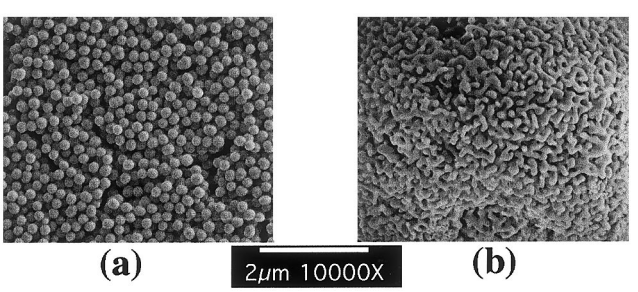

Fig. 3. Comparison of pore structutres between: (a) glass slide dip-coated with 200 nm silica microspheres (eight coats). Silica microspheres were formed via base catalyzed sol–gel process. (b) commercially available porous silica particle, 7 μm diameter, 100 nm pores.

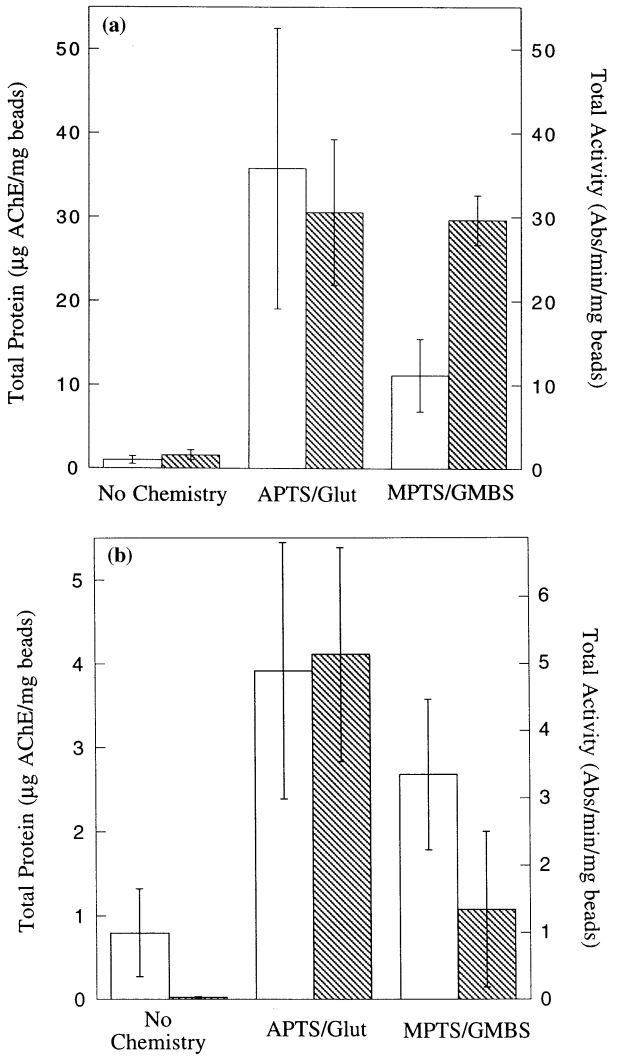

Fig. 4. Effect of immobilization chemistry on acetylcholinesterase loading (□) and total activity (▨) for porous and non-porous silica particles. (a) Porous particles; (b) non-porous particles.

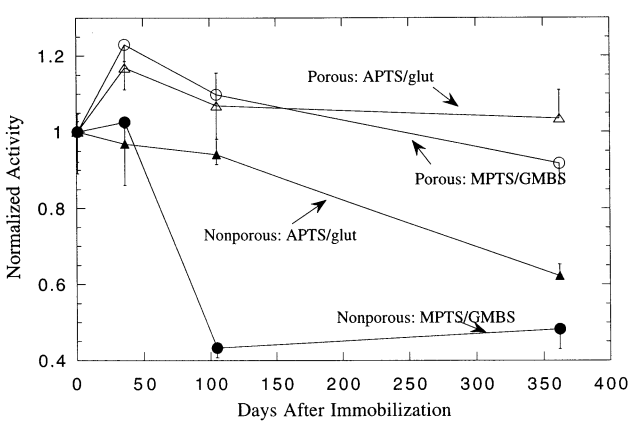

Fig. 5. Stability of acetylcholine esterase immobilized using two different chemistries to porous and non-porous silica particles. The total activity values have been normalized with respect to the total activity on day 1 for each bead/chemistry comination. APTS, 3-aminopropyltrimethoxysilane; glut., glutaraldehyde; MPTS, mercaptopropyltriethoxysilane; GMBS, *N*-γ-maleimidobutyryloxy succinimide ester.

tion of non-specific adsorption to the low levels presented in Fig. 4. For porous silica, the presence of 0.05% Tween-20, reduced the non-specific binding by 22%; in the case of non-porous particles, the reduction was 46%. Exposure to Tween-20 did not have any detrimental effect on the specific activity of enzyme. To reduce the electrostatic interactions, a solution containing high concentration of salt (0.5 M NaCl) was also used to wash the beads after reaction. The salt concentration was kept relatively high (0.14 M) also in the reaction mixture to minimize electrostatic interaction between protein and silica.

Fig. 5 shows the stability of immobilized AChE in terms of total activity over a period of 1 year for the different immobilization chemistries and supports. To facilitate qualitative comparison, the total activity has been normalized with respect to the total activity on day 1 for each combination of immobilization chemistry and bead type. For porous particles, regardless of immobilization chemistry, there is very little loss in enzyme activity over time. The results are remarkably different for non-porous particles. The total activity decreases by 38 and 52% over 1 year for particles coated with AChE using glutaraldehyde and GMBS, respectively.

3.3. Immobilization of organophosphate hydrolase

Fig. 6 compares the effectiveness of the two immobilization chemistries in terms of amount of OPH immobilized and total OPH activity for porous and non-porous silica particles. Again, the much greater surface area of the porous particles results in significantly greater enzyme loading and enzyme activity for porous particles compared with non-porous particles. For porous beads (Fig. 6a), the enzyme load and total activity was significantly less with the GMBS than with the glutaraldehyde, while the specific activity was approximately the same. For non-porous silica (Fig. 6b), the enzyme loading was equivalent for the two chemistries, while glutaraldehyde linkage performed better than GMBS in terms of total activity. The non-specific adsorption of enzyme (labeled 'No Chemistry' in Fig. 6a and b) is minimal in all cases, indicating that stringent washes and presence of Tween-20 in the reaction mixture minimizes interaction of enzyme with the silica surface. As observed in case of AChE, presence of detergent led to a much more dramatic reduction in non-specific adsorption for non-porous supports (70–125%) than porous supports (less than 7%).

Fig. 7 shows the stability of immobilized OPH in terms of total activity over a period of 1 year for the different immobilization chemistries and supports. The total activity has been normalized with respect to the total activity on day 1 for each combination of immobilization chemistry and bead type. As with the AChE,

for porous particles, regardless of immobilization chemistry, there is very little loss in enzyme activity over time. In fact, the total activity of immobilized enzyme increases up to 50% over 9 months. For non-porous particles there is a significant activity loss. Over 9 months, the total activity decreases by 57 and 36% for particles coated with OPH using glutaraldehyde and GMBS, respectively.

3.4. Comparison of APTS/glutaraldehyde and MPTS/GMBS immobilization chemistries

Both reaction schemes were successful in providing a good yield of immobilized enzymes. Furthermore, with both schemes, immobilized enzyme retained 25–50% of its specific activity compared with soluble enzyme (Table 1). Both glutaraldehyde and GMBS employ very mild pH conditions (7.4) and the reaction was carried out at 4°C to further reduce the possibility of denaturation during reaction. For both enzymes, the APTS/glutaraldehyde immobilization results in greater amounts of enzyme immobilized and greater total activity. This result may potentially be as a result of the generally accepted mechanism of glutaraldehyde self-polymerization (Richards and Knowles, 1968; Wong, 1993). Even though glutaraldehyde and protein are not added simultaneously, self-polymerization of glutaraldehyde can result in a polymerized multilayered enzyme film. This would result in greater enzyme loading. In addition, triethoxysilanes such as APTS, are known to polymerize in aqueous solutions leading to multilayer deposition on surfaces (Plueddemann, 1982). Multilayer silane deposition would also increase enzyme loading. The MPTS/GMBS immobilization is not subject to either of these issues and in fact, was specifically tailored to provide monolayer protein coverage. MPTS was deposited from dry toluene to avoid hydrolysis and self-polymerization; and, GMBS is a heterobifunctional crosslinker specifically designed to preclude homo-polymerization. It was expected that MPTS/GMBS would

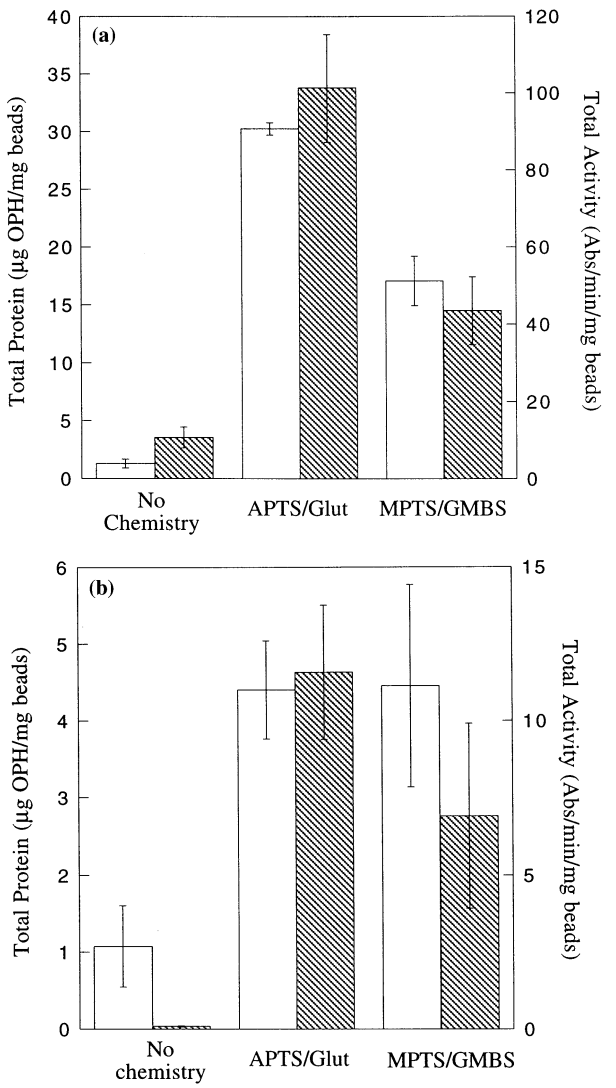

Fig. 6. Effect of immobilization chemistry on organophosphate hydrolase loading (□) and total activity (▨) for porous and non-porous silica particles. (a) Porous particles; (b) non-porous particles.

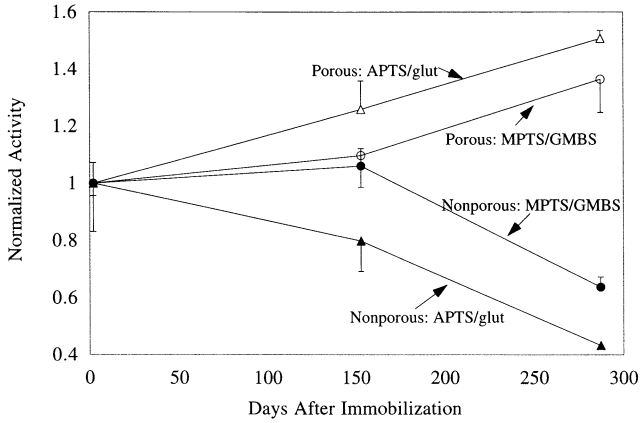

Fig. 7. Stability of organophosphate hydrolase immobilized using two different chemistries to porous and non-porous silica particles. The total activity values have been normalized with respect to the total activity on day 1 for each bead/chemistry comination. APTS, 3-aminopropyltriethoxysilane; glut., glutaraldehyde; MPTS, mercaptopropyltrimethoxysilane; GMBS, *N*-γ-maleimidobutyryloxy succinimide ester.

Table 1
Comparison of apparent K_m for organophosphate hydrolase immobilized to porous and non-porous silica beads[a]

Immobilization chemistry	Non-porous beads		Porous beads	
	K_m (mM)	k_{cat} (/s)	K_m (mM)	k_{cat} (/s)
MPTS/GMBS	0.048	204	0.44	343
APTS/glutaraldehyde	0.081	350	1.14	433

[a] For soluble enzyme, $K_m = 0.039$ mM, $k_{cat} = 857$.

provide better reproducibility with respect to mass and total activity of immobilized enzyme but comparison of standard deviations in Figs. 4 and 7 indicates no significant difference compared with APTS/glutaraldehyde scheme. In fact, run-to-run variations are relatively high for both chemistries for the two enzymes used, a problem encountered by a majority of biosensor investigations. In terms of sensor performance, total activity is the critical parameter of interest. Therefore, even though the MPTS/GMBS offers greater specific activity in some cases; overall, APTS/glutaraldehyde provided a superior performance.

3.5. Comparison of porous and non-porous matrices

For both enzymes the porous matrices were significantly superior in maintaining enzyme activity. In other words, enzyme immobilized on non-porous support is deactivated at a higher rate. It is possible that the porous structure provides an environment that protects enzyme from deactivation; pores protect protein molecules from shear forces that protein molecules on the outside surface are subjected to during mixing or contact with other particles. Another factor could be multipoint attachment of enzyme molecules inside the pores leading to higher degree of immobilization and hence better stabilization. This significant difference between porous and non-porous particles indicates a strong preference for sensors that provide a porous surface for immobilization.

Another issue in designing a biosensor using porous structures is diffusional resistance offered to substrate/analyte molecules. This can be evaluated by comparing the apparent K_m values for soluble and immobilized enzyme. For immobilized enzyme, K_m is strongly dependent on diffusional resistance. For non-porous particles, the enzyme is immobilized on the outer surface, and mass transfer resistance is limited to diffusion within the diffusion (or boundary) layer at the particle surface. However, for porous particles, there is an additional mass transfer resistance as a result of diffusion from bulk solution through the porous matrix. Table 1 provides a comparison of K_m for the OPH immobilization. As expected, K_m is lowest for soluble enzyme and the highest for enzyme immobilized in porous supports. There was virtually no increase in K_m for enzyme immobilized on non-porous silica using GMBS but a 2-fold increase in case of glutaraldehyde. The higher K_m and hence, the higher mass transfer resistance, for immobilization using glutaraldehyde chemistry supports the earlier suggestion that immobilization using APTS/glutaraldehyde may create a polymeric, multilayered matrix on the silica surface. For porous particles, enzyme immobilized using GMBS and glutaraldehyde showed an increase in K_m of 11- and 29-fold, respectively. Again, immobilization using APTS/glutaraldehyde leads to greater K_m than that using GMBS.

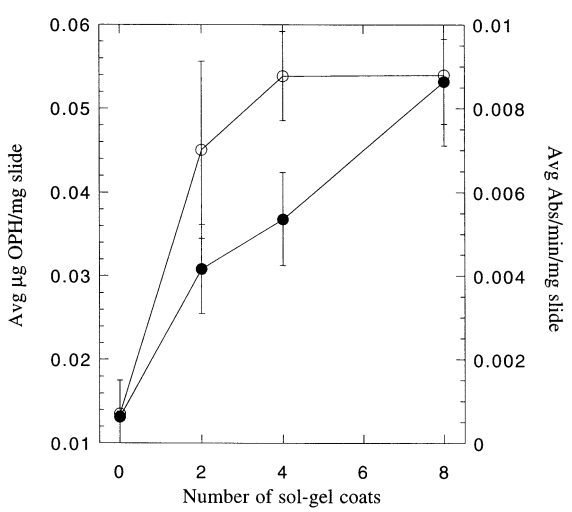

Fig. 8. Effect of number of sol–gel coats on OPH loading (○) and total activity (●). Thickness of one sol–gel coat is approximately 200 nm. Glass slides were dip-coated with 200 nm silica microspheres. Silica microspheres were formed via a base catalyzed sol–gel process. Samples were fired at 400°C in air for 10 min after each coat was applied.

3.6. Immobilization results with sol–gel coated substrates

To study the effect of sol–gel coating thickness on enzyme loading and activity, glass slides were coated with varying number of sol–gel layers. The APTS/glutaraldehyde reaction chemistry was used to immobilize enzyme to these samples. Fig. 8 shows the effect of number of coats of 200 nm sol–gel spheres on amount and total activity of immobilized OPH. As expected, both total activity and amount of immobilized enzyme increased as more sol–gel coats were deposited on the surface. However, the amount of enzyme immobilized increased linearly up to eight coats, while total activity reached a saturation point at four coats. This difference is likely as a result of diffusional resistance offered by the porous matrix to transport of substrate. In other words, more and more enzyme can be immobilized by increasing the number of sol–gel coats but a point is reached where substrate can not diffuse fast enough to keep up with the reaction rate. Based on this result, deposition of four coats was selected as the optimum coating to be applied to sensor surfaces.

It is also important to compare these results in terms of specific activity. Specific activity of sol–gel coated surfaces increased with number of sol–gel coats but most of the increase occurred between zero and two coats. Even with only two sol–gel coats a majority of the enzyme is immobilized within the pores and thus is protected from denaturing conditions such as shear

forces of mixing and proteolytic or bacterial attack. A portable device such as a field effect transistor will undoubtedly be exposed to harsh conditions in a field application, and presence of pores should protect immobilized enzyme molecules from a number of potentially harmful agents and conditions.

4. Conclusions

The sol–gel process is a well-established method for making porous ceramic structures. We employed a base catalyzed sol–gel process to form uniform silica microspheres. These microspheres were then dip-coated on planar silica surfaces to form a porous silica matrix to increase the surface area available for enzyme immobilization. Samples with sol–gel coats displayed significant increase in total enzyme activity. To further characterize the effect of porosity on enzyme immobilization, commercially available porous silica beads were used as analogs of sol–gel coated planar surfaces. Porous silica beads are manufactured by essentially the same method we employed to coat silica surfaces, and thus provide physically similar porous structure. Long term enzyme activity measurements indicated that porous matrices were also significantly superior in maintaining immobilized enzyme activity compared with non-porous substrates. No significant loss of enzyme activity was noted more than 12 months after enzyme immobilization.

Two different immobilization schemes were investigated to covalently attach AChE and OPH to silica beads and sol–gel coated slides. The first immobilization chemistry was the commonly used APTS/glutaraldehyde chemistry in which silica is amine-functionalized in aqueous media followed by protein attachment using the homobifunctional crosslinker glutaraldehyde. The second immobilization chemistry was silanization in anhydrous toluene to form a mercapto-functionalized surface followed by protein attachment using the heterobifunctional linker GMBS. Both chemistries resulted in good yields of immobilized enzyme and both appeared to be sufficiently mild to preserve up to half of the enzyme's specific activity. For porous matrices, the MPTS/GMBS chemistry offered greater specific activity but the APTS/glutaraldehyde scheme offered equivalent total activity. For non-porous matrices, the glutaraldehyde based chemistry was superior in terms of both total and specific activity of immobilized enzyme. APTS/glutaraldehyde was also the simpler, cheaper and more environmentally benign of the two methods, factors important in making mass-produced, inexpensive portable biosensors.

Based on these studies, the APTS/glutaraldehyde immobilization scheme was used to immobilize OPH to sol–gel modified field effect transistors as described in Flounders et al. (1999).

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