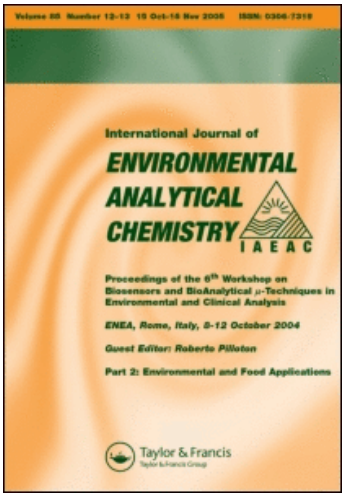

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MOLECULAR IMPRINTING OF SOL GEL POLYMERS FOR THE DETECTION OF PARAOXON IN WATER

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The molecular imprinting technique of the toxic organophosphate compound paraoxon was applied in thin films of organically modified silica (ORMOSIL) matrix. Paraoxon was imprinted in the ORMOSIL through π-π interaction and hydrogen or polar bonds originating from functional alkoxysilane monomers. The binding of paraoxon to the imprinted sol gel matrix was evaluated by inhibition of butyryl cholinesterase (BuChE) using the Ellman colorimetric assay. Two ORMOSILs that differ in the structure of the backbone monomer and functional monomers were investigated, and found to have similar specific binding properties. The kinetic profile of paraoxon binding to the polymer matrix was studied, and saturation was found to occur after *ca.* 2 h. The binding of paraoxon to the synthetic receptor was evaluated by application of the two site Langmuir analysis. Two classes of receptor sites were detected, with binding affinities of 0.04 and 7 nM, and site population of 57 and 25 nM, respectively.

INTRODUCTION

Organophosphate triesters are widely used as agricultural pesticides and herbicides. These compounds are very stable and can rapidly diffuse into ground water reservoirs and thus exhibit a threat of contamination. The common methods for the detection of low concentration of organophosphate pesticides are mainly based on chromatographic detection, in conjunction with element specific or mass spectrometry techniques [1]. These methods provide sensitive determination at the ppb levels of the organophosphate compounds, but they are very expensive and require skilled operators. In addition, these methods often require pretreatment of the sample, for example solid phase microextraction (SPME) [2] which is time consuming. A number of biosensors for organophosphate compounds based on interaction with surface-immobilized enzymes like acetyl cholinesterase and organophosphate hydrolase with electrochemical [3–5] or fluorescent detection [6,7] have been developed.

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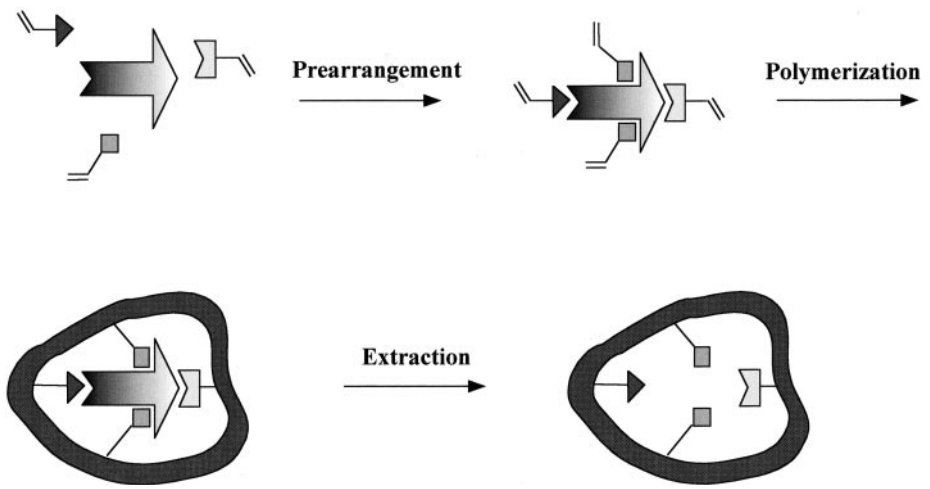

FIGURE 1 The principle of the molecular imprinting technique.

Selective binding combined with preconcentration can be achieved by using molecularly imprinted polymers. Molecular imprinting (MI) is a technique that has been evolving in the last two decades, and is used to create specific binding sites within a polymer matrix. The specific binding sites are realized by using functionalized monomers that interact with a template molecule through nonspecific interactions, Fig. 1. Polymerization with a high degree of cross linking and the subsequent removal of the template leaves a three-dimensional cavity in the polymer with complementary size and shape to the template molecule. The chemical functions that originate from the monomers situated in optimal position within the cavity to facilitate rebinding. MI has proved to be a useful technique in the development of selective chromatographic materials [8], microextraction fibers [9] and sensors [10–12]. The materials that are commonly used for MI are acrylic-based polymers. These polymers usually interact with the template molecule via hydrogen bonds, although electrostatic and hydrophobic interactions have been also demonstrated. Other polymer matrices that have been used for MI are polyurethane [13] and inorganic materials, in particular, sol gel [14] materials. The latter have been recently recognized as being superior to acrylic polymers in terms of adhesion to sensor surfaces and low nonspecific binding [15].

A number of studies have been performed in recent years on the ability to create specific binding sites for organophosphate materials by the molecular imprinting technique. For example, fiber optic sensors for organophosphate pesticides based on a 200 μm film of molecularly imprinted acrylic polymer that contained a fluorescent Eu^{3+} complex. The detection limit of this sensor was in the ppt range, with 15 min. response time [16]. Other studies dealt with creating catalytic binding sites by mimicking the active site of the enzyme organophosphate hydrolase [17], or using the tetrahedral phosphotriester as a transition state analog for the catalysis of carbamate ester hydrolysis [18]. All the aforementioned studies have been performed in acrylic polymer matrices. The application of acrylic polymers on sensor surfaces as thin films is complicated, and requires special polymerization conditions. In comparison,

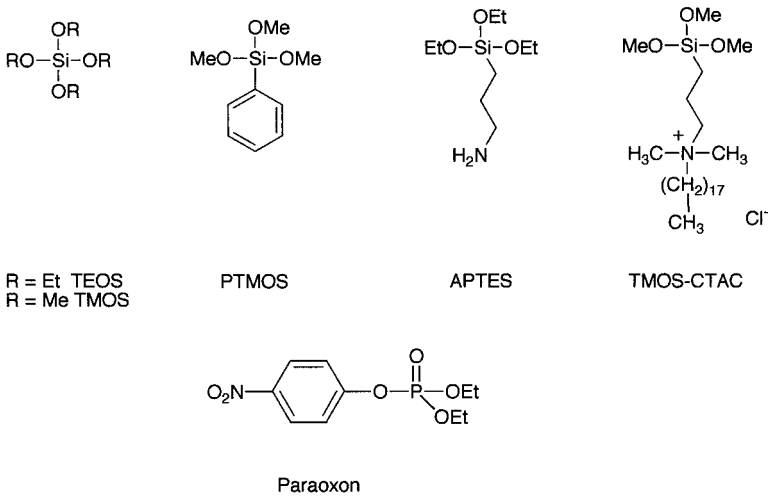

FIGURE 2 The chemical structure of the alkoxysilane functional monomers and the analyte used in the study.

the sol gel matrix is more suitable for the creation of thin films, and can be applied by spray, spin or dip-coating. The organically modified sol gel matrix was used to create specific binding sites for phosphonic acid by molecular imprinting [19,20]. Specific binding resulted from the electrostatic interaction between the phosphonic acid and a guanidiniun-modified monomer in basic medium.

Paraoxon is the toxic metabolite of the pesticide parathion and is formed in the body by oxidative desulfurination of the P–S bond of parathion by the microsomal P_{450} enzymes [21]. The toxicity of paraoxon is based on the irreversible inhibition of acetylcholine esterase enzyme due to the formation of a stable complex between the enzyme and the inhibitor. Hydrolysis of acetylcholine, the natural substrate for acetylcholine esterase is inhibited, and toxic effects occur due to the accumulation of acetylcholine.

In the present study, specific binding sites for paraoxon were molecularly imprinted in a thin film of sol gel polymer. The binding of paraoxon was evaluated by the level of inhibition to butyrylcholinesterase (BuChE) using the Ellman Assay [22]. In this study, two combinations of monomers were investigated (see Fig. 2 for the functional monomers that were used). The difference between the two combinations was the backbone monomer, tetraethyl orthosilicate or tetramethyl orthosilicate, which determines the hydrolysis rate, and the functional monomers that interact with paraoxon through noncovalent interactions.

EXPERIMENTAL

Chemicals

Tetraethyl orthosilicate (TEOS) 99+%, tetramethyl orthosilicate (TMOS) 99+%, aminopropyltriethoxysilane (APTES) 99%, phenyl trimethoxysilane (PTMOS) 97%, [3-(trimethoxysilyl)propyl] octadecyldimethylammonium chloride, TMOS-CTAC

(72% in ethanol), 2-ethoxy ethanol were from Aldrich. Bovine serum albumin (BSA) fraction V, *S*-butyryl thiocholine chloride (BTC), 5,5′-dithio-bis(2-nitrobenzoic acid) (DTNB), and HEPES buffer were from Sigma. Paraoxon was from Supelco. 10 mM phosphate buffer was prepared by mixing equimolar amounts of K_2HPO_4 and KH_2PO_4 (Merck) in distilled water and titrating to pH 7.6 with 0.1 M KOH. Horse butyryl cholinesterase (BuChE) (E.C.3.1.1.8, 28 u/mg) was from Biozyme UK. All other chemicals and solvents were of analytical grade.

Preparation of Molecularly Imprinted Sol Gel Films

TEOS Based Films (P1-PO): TEOS (3 mL, 13.5 mmol), PTMOS (200 μL, 1.2 mmol) and ethanol (3 mL) were mixed until the solution was clear. 100 μL of concentrated HCl was carefully added followed by 200 μL (0.9 mmol) of APTES and 1 mL of H_2O. 2 mL of the sol was mixed with 200 μL of 0.1 M (0.02 mmol) ethanolic solution of paraoxon for the imprinted sol. After 24 h of mixing, the aged sols were used to spin coat glass surfaces (cover glass 13 mm in diameter, BDH). Spin coating was performed on Headway ECOID 101 spin-coater (4000 rpm for 20 s), by placing 30 μL on the glass surface. The thickness of the film was 534 ± 6 nm (measured by Filmetrics F-20 instrument on silicon wafer coated with the same sol). Reference films (P1-B) of the nonimprinted gel were also prepared in the same manner.

TMOS Based Films (P2-PO): TMOS (3 mL, 20.3 mmol), 2-ethoxy ethanol (3 mL), PTMOS (370 μL, 2.2 mmol) and TMOS-CTAC (420 μL, 0.6 mmol) were mixed. After a clear solution was formed, 1 mL of 0.1 M HCl and 1 mL of H_2O were added. The sol was stirred for 2 h at RT, and 2 mL was mixed with 200 μL of 0.1 M ethanolic solution of paraoxon for the imprinted sol. The sol was then used immediately for spin coating, as described above.

After drying of the gels (P1-PO and P2-PO) for 24 hr, paraoxon was extracted from the imprinted gels by sohxlet extraction with ethanol. Nonimprinted gels (P1-B and P2-B) were also subjected to Soxhlet extraction to remove unreacted material.

Paraoxon Binding Assay: The assay was based on the measurement of the residual activity of BuChE after inhibition with bound paraoxon by the Ellman assay. The coated glass plates were incubated in 2 mL of aqueous solution of paraxon, concentrations $1\,\text{nM} - 0.1\,\text{mM}$, in 10 mM HEPES buffer, $\text{pH} = 7.8$ at 25°C with gentle shaking. The duration of the incubation was usually 3 h, except for the kinetic profile experiment. After the incubation, the glass plates were rinsed by dipping into clean buffer solution ($\times 4$ times) to remove physically adsorbed material. Each plate was then placed in a well of 24-well polystyrene microtiter plate (Costar). 200 μL of BuChE (2.3 nM in 0.5 M HEPES buffer, $\text{pH} = 7.8$ containing 0.1% w/v BSA) was added to each plate, and then incubated for 20 min at 30°C. During this time period, the bound paraoxon diffuses out of the binding cavities into the buffer solution and binds covalently to the enzyme. The nonequilibrium conditions assure that all of the bound paraoxon will bind to the enzyme. Preliminary experiments were performed to ensure complete leaching of paraoxon from the polymer. After the incubation period, 200 μL of a PB solution containing 1 mM of BTC and 0.6 mM of DTNB in phosphate buffer, 0.5 M ($\text{pH} = 8$) were added to each well. The absorbance at $\lambda = 405$ nm was recorded by ELISA reader (Tecan, Spectrafluor plus), and

the residual activity of the enzyme was calculated from the initial velocity of the enzymatic reaction. The concentration of bound paraoxon was calculated by comparing to a calibration graph, prepared by incubating the same concentration of BuChE with solutions of paraoxon in the same conditions, and calculating the concentration resulting from the residual activity according to Eq. 1:

$$\text{paraoxon nM} = 116 \times \left(\frac{\text{rate}_{\text{BuChE+paraoxon}}}{\text{rate}_{\text{BuChE}}} - 0.95 \right) \quad (1)$$

where $\text{rate}_{\text{BuChE}+\text{paraoxon}}$ is the rate of the enzymatic reaction (OD/min) of the enzyme incubated with paraoxon and $\text{rate}_{\text{BuchE}}$ is the rate of the enzymatic reaction (OD/min) of the native enzyme, in the absence of paraoxon. Each experimental data point is the mean of 4 plates. In each experiment, the uptake of paraoxon by paraoxon-imprinted sol gel polymer (P1-PO and P2-PO) was compared to the uptake of the reference nonimprinted polymer (P1-B and P2-B).

RESULTS AND DISCUSSION

Effect of the Polymer Matrix

Two sol gel materials were imprinted with paraoxon. TEOS based sol gel is known to hydrolyze more slowly relative to the more reactive TMOS based, under acidic hydrolysis conditions. The functional monomers in both matrices contained a phenyl group, which interact with the nitrophenyl group on the paraoxon via $\pi - \pi$ interaction, and an amine group (primary amine for P1 and quaternary amine for P2). The primary amine is to a certain extent capable of establishing hydrogen bonds with the phosphate group, mainly with the P = O bond, while the interaction of the quaternary amine with paraoxon is probably based on polarization interactions. The quaternary amine monomer, TEOS-CTAC contained also a long alkyl chain, and is essentially a combination of a trialkoxy silane with a surfactant. The addition of surfactants as additives (in contrary to the preparation of mesoporous silica by surfactant templating techniques) to sol gel material had been developed recently. It was found that the addition of surfactants can modify the physical and chemical properties of the sol gel, and specifically can influence the chemical microstructure of the pores [23].

The two imprinted polymers were subjected to uptake experiments with 10 μM of paraoxon. The binding of paraoxon by both imprinted polymers and reference polymers is illustrated in Fig. 3. The specific binding of paraoxon by P1-PO and P2-PO is similar, 0.18 ± 0.05 and 0.16 ± 0.05 pmole, respectively. The nonspecific binding which is measured by the uptake of the nonimprinted polymers (P1-B and P2-B) was low, 0.022 ± 0.003 and 0.0028 ± 0.004 pmole, respectively. The variation between the different plates, mostly due to minute difference in film thickness, induces the relatively high deviation in the results.

It is interesting that both polymer compositions possessed the same recognition properties. This may point to the fact that one of the more important factors that govern the specific binding is the presence of functional groups in the binding cavity and that the role of the backbone monomer (TEOS or TMOS) is of lesser importance.

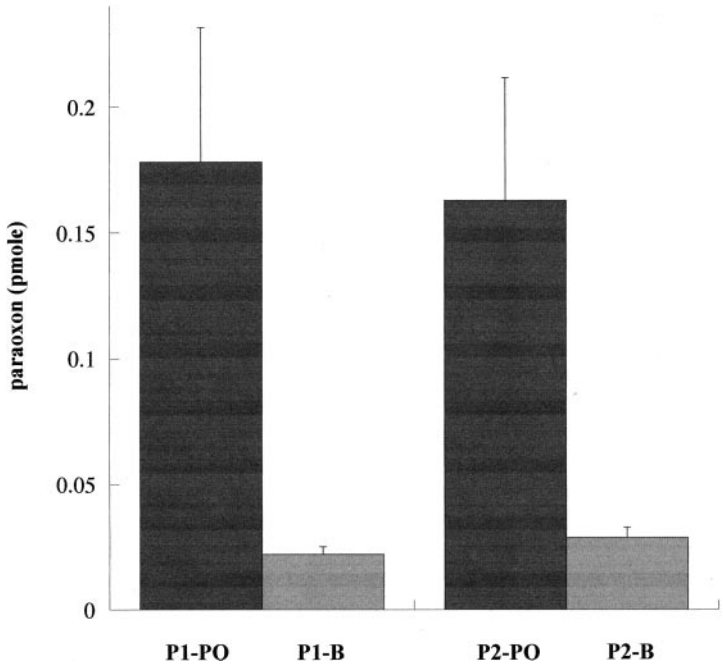

FIGURE 3 Steady state binding of paraoxon (3 h incubation in 10 μM paraoxon in 10 mM PB pH = 7.6 at 25°C) to P1 and P2. Black bars represent binding to paraoxon imprinted polymers, P1-PO and P2-PO, and gray bars represent binding to nonimprinted polymers, P1-B and P2-B. ($n = 4$).

The questions of the role of the functional monomer and synergistic effects were explored in greater detail and will be reported in the future.

Kinetics of Binding

The diffusion of paraoxon into the polymer is dictated by the pore structure of the polymer [24]. The pore structure is controlled by various factors, like the nature of the precursors, their concentration, amount of water present, pH, temperature and solvents. TEOS-based sol gels are known to be more porous than TMOS bases [25].

The kinetic profile of the uptake of paraoxon by polymer P1 is illustrated in Fig. 4. The specific binding of paraoxon by the imprinted polymer is apparent after a short incubation period of 10 min. The nonspecific binding remains low throughout the experiments, and saturation is achieved after *ca.* 2 h. The long saturation period is probably the result of the lower porosity of the thin film, relative to the bulk [26].

Binding Properties

The ability of the imprinted polymer P1 to absorb paraoxon was investigated in a wide range of concentration, Fig. 5. While the reference polymer P1-B exhibits low non-specific binding all through the concentration range investigated (0.5 nM − 0.1 mM), the specific binding is apparent in a concentration as low as 0.5 nM, and saturation is reached at 1 μM of paraoxon. This binding profile was used to calculate the binding affinity of paraoxon to the synthetic polymer receptor using the two site Langmuir

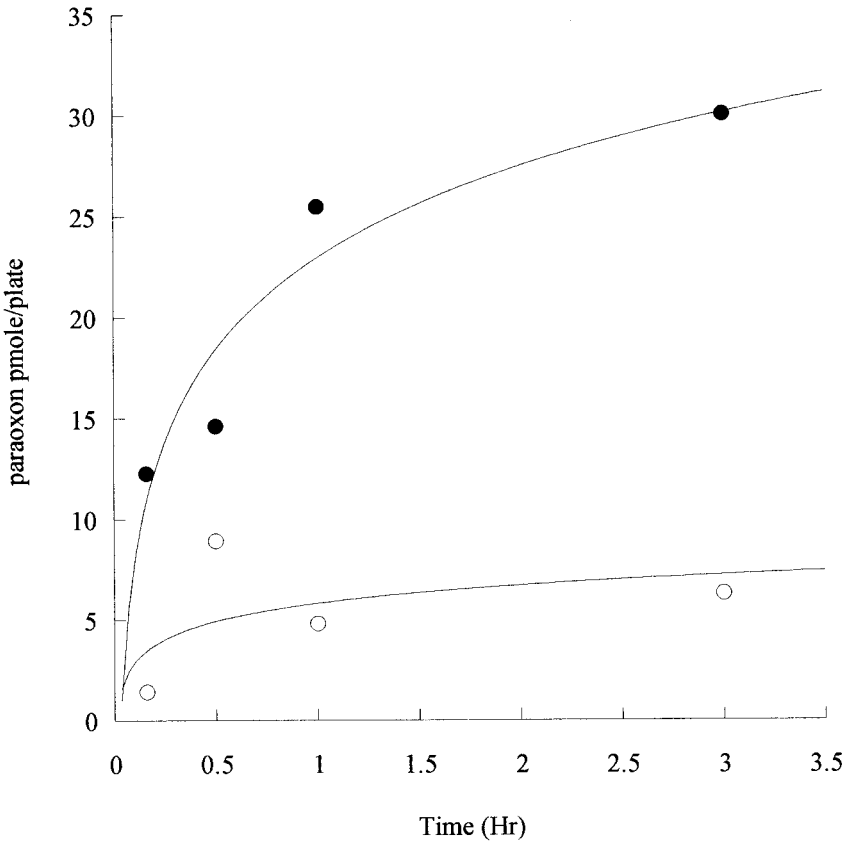

FIGURE 4 Kinetic uptake profile of paraoxon by P1-PO (full circles) and PI-B (open circles). Samples were incubated in 10 μM paraoxon in 10 mM PB, pH = 7.6 at 25°C ($n = 4$).

analysis [27]. Figure 6 exhibits match of the two site Langmuir equation to the experimental data points. The bi-phasic plot, characteristic of synthetic receptors prepared by the molecular imprinting technique [28–30]. The nonlinearity is due to heterogeneous population of binding sites, where the low concentration component of the Langmuir plot corresponds to the highly specific binding sites, that bind paraoxon with high affinity, $K_d = 0.04$ nM. The high concentration component is attributed to the less specific binding sites, which in the present case possess also very high affinity towards paraoxon, $K_d = 7$ nM. The maximum site populations that correspond to these binding affinities are 57 and 25 nM, respectively. The heterogeneous nature of the binding sites is due to the noncovalent interactions between the functional monomers and the template molecule. The association between the template and the functional monomers in the multi component complex that exist prior and during polymerization is not completely defined, and in some cases can rely on several modes of interaction. The molar ratio between paraoxon, PTMOS and APTES in the P1 sol gel polymer is 1 : 60 : 40, so it is possible that in some cavities the nitrophenyl group of paraoxon is bound to one phenyl group of PTMOS, while in other cavities the nitrophenyl group is stacked between two phenyl groups. Both complexes will

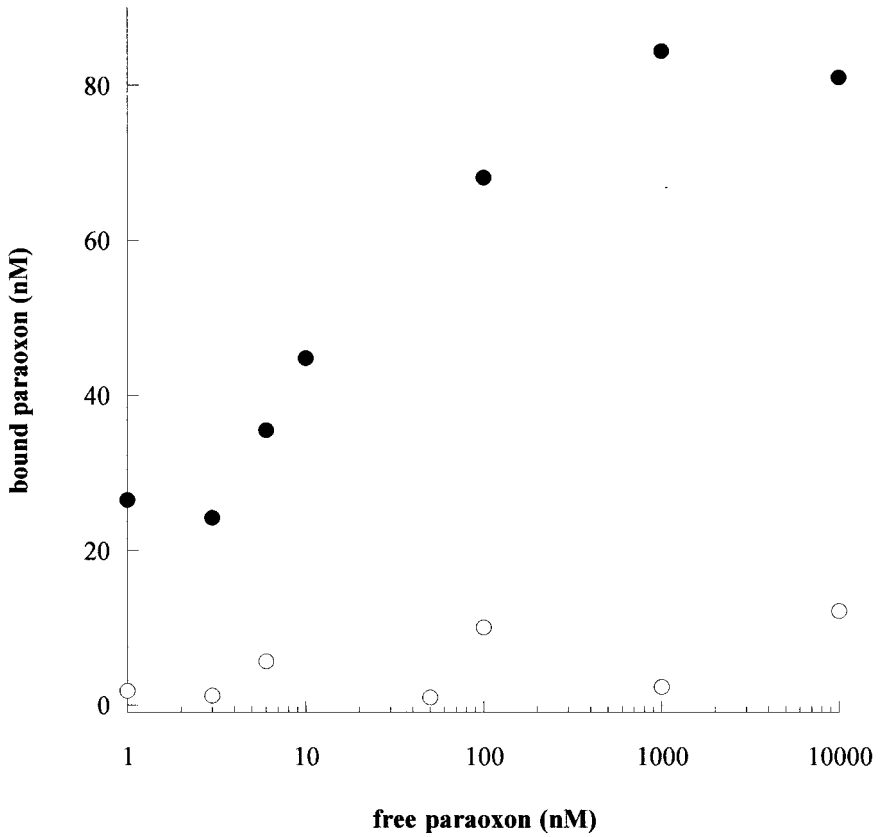

FIGURE 5 Paraoxon saturation binding profile to P1-PO (closed circles) and P1-B (open circles), after incubation of 3 h in paraoxon solutions in 10 mM PB pH = 7.6 ($n = 4$).

yield specific binding, as apparent from the high association constants. The heterogeneity of the binding sites can be eliminated by using the covalent approach for molecular imprinting [31]. However, this approach requires the preliminary synthesis of a cleavable monomer-template compound. It should be noted that the association constant of paraoxon to the imprinted polymer is in the range of antibody binding. Since, to our knowledge, there is no antibody for paraoxon, this polymer may serve as a basis for a binding assay for paraoxon or related compounds.

CONCLUSIONS

The specific binding of paraoxon in molecularly imprinted thin films of organically modified sol gel materials has been demonstrated. The detection method, based on BuChE inhibition, provided a very sensitive method for the detection of pmole amounts of bound paraoxon into the thin film in relatively short assay time. Nonspecific binding was negligible in comparison with the specific binding which could be monitored in a broad range of concentrations. It was also shown that the crea-

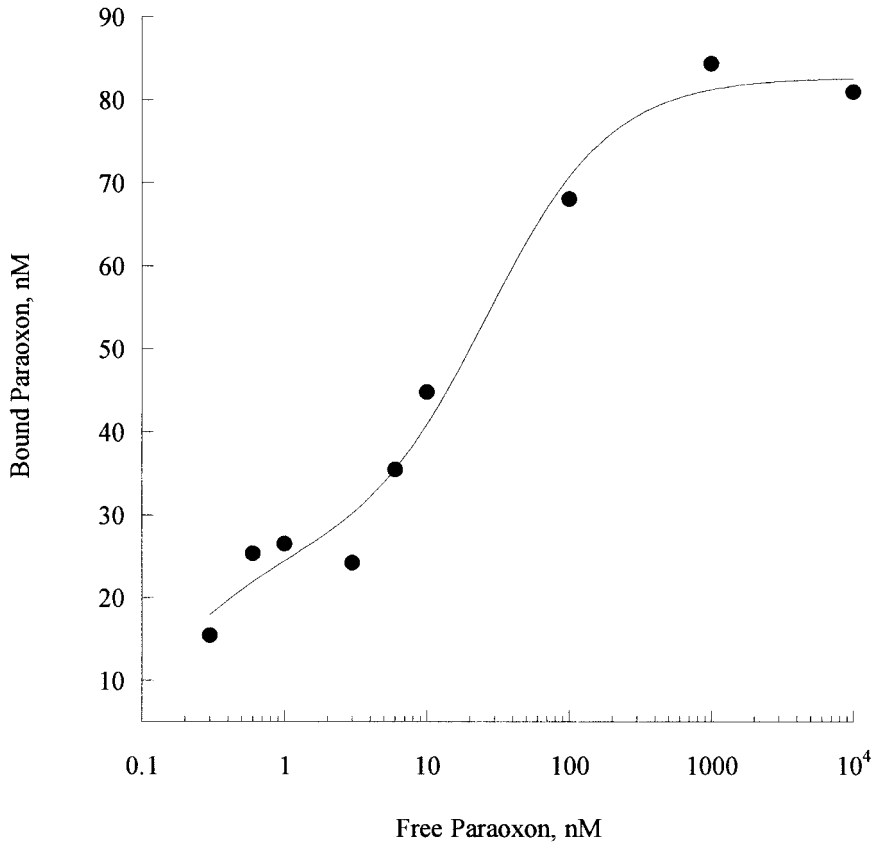

FIGURE 6 Two site Langmuir analysis for the binding of paraoxon to sol gel P1-PO. $R^2 = 0.99$.

tion of a specific binding site is not limited to an optimal composition of functional monomers, but the same recognition properties can be realized using different functional monomers. Thus, it is argued that the binding is dependent mainly on the existence of noncovalent interactions between the template molecule and the chemical functions of the monomer, but this is not highly sensitive to the exact nature of the non-covalent interactions that exist between the template and the monomers. The molecularly imprinted thin films can be easily coupled to a variety of transduction systems, such as QCM, SPR, electrode surfaces or fiber optic sensors, to provide stable, sensitive, reversible and cheap recognition layers for small molecules, where natural receptors or antibodies are difficult, or impossible to produce. Another possible application is the development of target-specific microextraction devices. The reversibility of the binding is based on the extraction of the bound target molecule from the polymer by a solvent, similar to the extraction of the template molecule from the freshly imprinted polymer. The use of the organically modified sol gel matrices opens a range of applicable matrix configurations – thin films, monoliths, beads etc., that can be used in sensors, antibody replacements in assays, selective chromatography columns and microextraction devices. Initial reports on the use of molecular imprinting in sol gel matrices show great promise in the use of this combined technique in many research areas.

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