

SELECTIVE MOLECULARLY IMPRINTED POLYMERS FOR FLUOROPHOSPHONATE NERVE GASES – PROOF OF PRINCIPLE

Bernard S. Green*, Alexander G. Strikovskiy, Inna Pergament-Tzomik, Rina Arad-Yellin and Yacov Ashani

Semorex Inc. 675 US Highway One; North Brunswick, NJ 08902 USA and Semorex Technologies Ltd., Bldg. 7; Weizmann Science Park, PO Box 4021; Ness Ziona, Israel 70400 [*email: green@semorex.com]

Introduction

With the rise of urban terror threats and the potential for accidental release of toxic organophosphate (OP) nerve agents and pesticides, there is an increasing demand for field-rugged, hand-held sensor devices for detection and identification of OPs. The release of OP-based toxic chemicals demands quick and effective agent detection in order to allow decision makers to conclude whether to evacuate populations, to decontaminate and how to administer appropriate, prompt medical treatment. Significant work, directed to selective OP detection using non-covalent molecularly imprinted polymers (MIPs), has been reported¹⁻⁵. However, these displayed relatively weak binding and limited selectivity. We describe a novel approach for selective molecular recognition and sensing of OP nerve agents containing a P-F bond (e.g., sarin, cyclosarin, soman), envisaged to be superior to the less specific and unstable biological-based field detection for OP exposure based on cholinesterase inhibition. Our MIP design creates selective binding site cavities, complementary to the spatial configuration of the nerve agent (e.g., sarin), and project a strong nucleophile (hydroxamic acid) to the bound OP agent so as to efficiently attack the phosphorous-fluoride bond. This also produces a fluoride ion that can trigger a quantifiable signal⁶. Our approach is illustrated in **Figure 1**. Four MIPs were synthesized and tested against five OP model compounds, providing the first proof-of-concept for this approach. The incorporation of functional active groups into MIPs provides potential development paths to diverse field detection devices that are based on chemical recognition rather than biological activity.

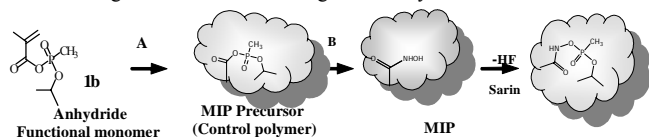


Figure 1. Semorex design for obtaining strategically functionalized MIP binding sites involves polymerization of anhydride functional monomers (methacryl isopropyl methylphosphonyl anhydride, **1b**, for sarin-binding MIP) and treating the resulting highly cross-linked macroporous polymers (control polymers for the corresponding MIP) with hydroxylamine. This reaction releases the phosphonic acid, creating a complementary binding cavity, and also forms the hydroxamate nucleophile. Conditions: A: 95% cross-linker, solvent (porogen), initiator, heat; B: excess hydroxylamine in methanol.

Experimental

Instrumentation. A UV Microplate Spectrophotometer (PowerWave XS, Bio-tech) was used to monitor p-nitrophenol optical densities in kinetic measurements. ¹H and ³¹P NMR spectra of the functional monomers and substrates were recorded on a Bruker DPX-250 MHz spectrometer.

Synthesis of functional monomers and substrates. Functional monomers based on mixed anhydrides of carboxylic-phosphonic (or phosphoric) acids were synthesized as shown in **Figure 2**. All products were purified by column chromatography on silica and were fully characterized by ¹H and ³¹P NMR spectra and by mass spectroscopy. A representative synthetic protocol, for the functional monomer depicted in **Figure 1**, follows: To one equivalent of distilled methyl dichlorophosphonate was added one equivalent of isopropyl alcohol and the reaction was allowed to proceed for 20 hr. The product, methyl isopropylchlorophosphonate, was distilled (64% yield) and reacted with equimolar amounts of methacrylic acid and triethyl amine. The product was purified on a silica gel column (petroleum ether/Et₂O as eluent) and isolated in 15% yield. ¹H NMR (CDCl₃, δ, ppm): 1.33 (6H, m), 1.74 (3H, d), 1.93 (3H, s), 4.88 (1H, m), 5.78 (1H, s), 6.23 (1H, s). ³¹P NMR: 25.11.

Data for the other functional monomers: **1a**: (23% yield) ¹H NMR: 1.37 (12H, t), 1.96 (3H, s), 4.87 (2H, q), 5.79 (1H, s), 6.23 (1H, s). ³¹P NMR: -8.43. **1c**: ¹H NMR: 0.92 (9H, s), 1.77 (3H, d), 1.93 (3H, s), 4.47 (1H, m), 5.78 (1H, s), 6.23 (1H, s). ³¹P NMR: 25.17, 25.49. **1d**: ¹H NMR: 1.24-1.72 (11H, m), 1.75 (3H, d), 1.95 (3H, s), 4.68 (1H, m), 5.78 (1H, s), 6.23 (1H, s). ³¹P NMR: 28.08.

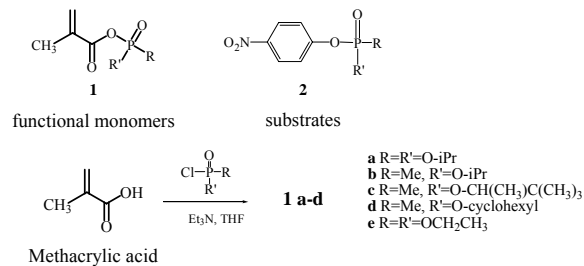


Figure 2. Molecular Structures of the synthesized functional monomers and of the substrates studied.

The p-nitrophenyl phosphonate substrates, **2b-d** were prepared from the corresponding chlorophosphonate derivatives using p-nitrophenol, and NaH. Similar reagents were used to prepare **2a** starting from diisopropyl chlorophosphate. NMR and MS spectra corroborated the structures. Substrate **2e** (paraoxon) is a commercial compound. The synthetic reaction conditions are as follows: p-nitrophenol (1 equivalent) was dissolved in dry THF and 1.1 equivalents of sodium hydride (95%) was added in small portions at 0 °C. The mixture was stirred for 0.5 hr followed by slowly adding phosphorus chloride (1 eq) at 0 °C, warming to room temperature and stirring for 2 hr. The solution was filtered, the solvent was evaporated and the residue was chromatographed on silica gel (petroleum ether/ethyl acetate eluent) to afford the product. Final purification of the products from p-nitrophenol was achieved by filtration over a short basic aluminum oxide column (yields from 17% to 23%). **2a**: ¹H NMR: 1.35 (12H, m), 4.79 (2H, m), 7.39 (2H, d), 8.24 (2H, d). ³¹P NMR: 7.45. **2b**: ¹H NMR: 1.25 (3H, d), 1.38 (3H, d), 1.66 (3H, d), 4.84 (1H, m), 7.38 (2H, d), 8.23 (2H, d). ³¹P NMR: 28.59. **2c**: ¹H NMR: 0.91 (12H, m), 1.75, 1.34 (3H, d), 1.26 (1H, d), 1.66 (3H, d), 7.41 (2H, d), 8.23 (2H, d). ³¹P NMR: 28.8 (d). **2**: ¹H NMR: 0.87 (1H, d), 0.94-1.54 (9H, m), 1.73 (3H, d), 4.54, (1H, m), 7.37 (2H, d), 8.22 (2H, d). ³¹P NMR: 28.62.

Synthesis of MIPs. The imprinted polymers were prepared as follows: the functional monomer (5% by weight) redistilled EGDMA (ethylene glycol dimethacrylate, cross-linker, 95% by weight) and AIBN (azo-isobutyronitrile, initiator, 1% by weight) were dissolved in toluene (3/4 of monomer volume). After three freeze-thaw cycles, the ampoule was heated (70 °C, overnight). The resulting polymer (92% yield) was crushed and washed with toluene, followed by MeOH, and dried at 100 °C for 24 hrs. The polymers were swelled in toluene and approx.1 equivalent (by weight) of freshly prepared hydroxyl amine in methanol was added. The mixture was stirred for 48 hours; the resulting MIP was washed, stirred with water (200 ml/g polymer), filtered and similarly washed with MeOH (200 ml), toluene (100 ml), and dried at 100 °C. The MIPs DFP-MIP, Sarin-MIP, etc. by virtue of their design to bind DFP, sarin, etc; thus, **Figure 1** depicts a sarin-MIP synthesis.

Determination of substrate hydrolysis rates, capacities and pKa of MIPs. Hydrolysis of substrate (in the presence of MIPs and, simultaneously, spontaneous hydrolysis in buffer, pH 10, NaHCO₃, 50 mM) was studied by monitoring the rate of p-nitrophenol release (at 405 nm). Typically, 20 mg of MIP or MIP control was incubated in the substrate solution (3 ml); Samples were withdrawn at various time intervals and the absorption was measured by a UV Microplate Instrument. For MIP capacities, different initial concentrations of substrate in buffer were used. The pKa value was determined by incubating MIP samples in solutions of NP-DFP (**2a**) at different pH values, obtained with sodium bicarbonate and phosphate buffers. The experimental data were calculated using Graphpad Prism 4.03 program software.

Results and Discussion

Synthesis. The synthesis of the anhydride functional monomers, **1 a-d**, was the critical initial step in this research. Our early attempts to achieve OP-selective nucleophile-positioned MIP cavities were based upon covalent nucleophile-phosphonate functional monomers, e.g., 4-vinylbenzaldehyde

oxime phosphoryl esters, hydrolyses of which were difficult. The mixed anhydrides are an excellent compromise between the required stability (sufficiently stable to be isolated and purified on silica columns) and lability, reacting smoothly with hydroxylamine even after polymerization to form MIPs, as depicted in **Figure 1**.

MIP capacity and pKa evaluation. The amount of active hydroxamic acid groups in the MIP was evaluated measuring p-nitrophenol produced after reaction of excess substrate with polymer-containing hydroxamic acids. The substrate hydrolysis in the presence of MIP was corrected for spontaneous substrate hydrolysis in buffer by subtracting the p-nitrophenol produced in buffer from that produced in the presence of MIP-buffer suspensions at the same corresponding times. Notably, increasing the substrate concentration increased the capacity, indicating the heterogeneity of the nucleophiles, hydroxamates, in the heterogeneous population of MIP binding sites (see **Figure 3**). MIP capacities for paraoxon (**2e**), NP-sarin (**2b**) and NP-soman (**2c**) substrates are shown in **Table 1**.

Table 1: Capacity and Kinetic Parameters for MIP-Assisted Hydrolysis of Paraoxon (2e), NP-Sarin (2b) and NP-Soman (2c).

MIP	MIP for corresponding substrates* ($\mu\text{mol/g}$)	MIP nucleophilicity**	
		Activity per g of MIP (nmol/g min)	Activity per nucleophilic center (mmol/active center_min)
DFP-MIP	22.1	5.1 \pm 1.2	0.23 \pm 0.05
Sarin-MIP	18.3	53	2.9
Soman-MIP	9.8	4.8	0.48

* Measured using the corresponding substrates, NP-sarin (**2b**) with Sarin-MIP and NP-soman (**2c**) with Soman-MIP, but paraoxon (**2e**), with DFP-MIP.

** Corrected for spontaneous substrate hydrolysis in buffer

A typical titration curve, obtained for NP-DFP (**2a**), is shown in **Figure 3a**. The velocity-pH dependence indicates a pKa value for the reactive MIP-hydroxamates of 10, which is consistent with the pKa of aliphatic hydroxamic acid. From the shape of the sigmoidal curve and the pKa value, it appears that the hydroxamic groups do not interact with one another but react independently with the substrate. The relatively high pKa value required our working with the MIPs at pH 10. Future MIPs can readily be designed to lower the pKa of the hydroxamate or other nucleophile and allow effective reaction at lower pH.

MIPs selectivity evaluation. The MIP-accelerated hydrolysis was expressed as a ratio of initial kinetic slope of substrate hydrolysis in the presence of MIP to initial kinetic slope of substrate hydrolysis in buffer. A ratio higher than 1.0 indicates acceleration of substrate hydrolysis by MIP compared to simultaneous hydrolysis in buffer. Results are summarized in **Table 2**.

Table 2. MIPs Selectivity Expressed in Initial Kinetic Velocity Ratios between MIP Facilitated Substrate Hydrolysis and Substrate Hydrolysis in Buffer.

MIP \ Substrate	DFP-MIP	Sarin-MIP	Soman-MIP	Cyclosarin-MIP
2e	1.6 \pm 0.1	1	0.9	-
2b	1.5	1.6	1	-
2c	0.9 \pm 0.2	1	1.4	-
2d	0.7	--	0.7	0.4

MIPs imprinted to react with DFP (DFP-MIP), soman (soman-MIP) and sarin (sarin-MIP) demonstrated marked selectivity by preferential hydrolysis of the corresponding NP substrates (**2b-2e**). DFP-MIP also facilitated hydrolysis of NP-sarin, but with a slightly slower rate compared with that for sarin-MIP. However, MIPs imprinted to react with cyclosarin (cyclosarin-MIP) did not accelerate NP-cyclosarin hydrolysis above its spontaneous hydrolysis rate and did not show selectivity for NP-cyclosarin hydrolysis in comparison to DFP- and soman-MIPs. The reasons for the anomalous behavior of cyclosarin-MIP are not yet clear and are under study.

Another problem that has to be addressed is the surprising lower activity of the control MIPs (data not shown) relative to the buffer. Although a comparison of MIP with control MIP would portray more dramatic acceleration, we preferred to use the buffer control since this is more appropriate to the real-life field situation. Apparently, the polymer matrix binds either the product or the reactant and this is lowering the apparent MIP reactivity. Further assessment of this phenomenon, using other polymerization methods merits study.

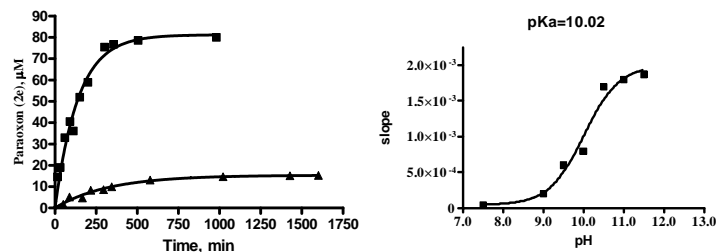


Figure 3. Left: determination of DFP-MIP capacity using paraoxon (**2e**). The ordinate indicates release of p-nitrophenol at pH 10, corrected for spontaneous **2e** hydrolysis in buffer; \blacksquare , \blacktriangle , 2.3 and 0.4 mM initial concentrations of **2e**, respectively. Right: determination of the pKa of the hydroxamate groups in DFP-MIP; the slope represents the initial velocity of p-nitrophenol release from paraoxon (**2e**).

Conclusions

Although further research is required in order to optimize the performance of the polymers, the marked selectivity and reactivity of these robust MIPs using model compounds is noteworthy and promises to find application in diverse areas. We await results of the four MIPs with the actual nerve gases, sarin, cyclosarin and soman, and with DFP (preliminary studies with DFP also indicate higher reactivity with the DFP-MIP in comparison with other MIPs); these studies are being carried out in collaboration with Dr. Steve Harvey, ECDC, Aberdeen Proving Ground, MD.

Acknowledgement. We thank Prof. Guenter Wulff (Heinrich Heine University, Duesseldorf) and Dr. Richard K. Gordon (Walter Reed Army Institute of Research) for valuable advice and contributions and Ms. Svetlana Firsov for expert technical assistance. This research was performed under US DoD Contract DAMD17-02-C-009.

References

- Parker, J. L., S. N. Horne, J. M. Kita, C. J. Peebles and J. C. DiCesare, Development of a sensor for the hydrolysis product of the nerve agent Soman utilizing molecular imprinting and silica sol-gel techniques, *Abstr. Pap. Am. Chem. Soc.* **2003**, 225, 198-PMSE.
- Reynolds, J. G. and B. R. Hart, Nanomaterials and their application to defence and homeland security, *JOM*, **2004**, 56, 36-39.
- Boyd, J. W., G. P. Cobb, G. E. Southard and G. M. Murray, Development of molecularly imprinted polymer sensors for chemical warfare agents. *Johns Hopkins Apl Technical Digest*. **2004**, 25, 44-49.
- Zuo, Y. J., J. H. Yu, Q. B. Huang and Y. Lin, The preparation of molecularly imprinting nanometer layer and its application in detecting nerve agent sarin. *Chin. J. Anal. Chem.* **2003**, 31, 769-773.
- Jenkins, A. L., O. M. Uy, G. M. Murray, Polymer-based lanthanide luminescent sensor for detection of the hydrolysis product of the nerve agent soman in water, *Anal. Chem.* **1999**, 71, 373-378.
- Mizuno, T., W. H. Wei, L. R. Eller, J. L. Sessler, Phenanthroline Complexes Bearing Fused Dipyrrolylquinoxaline Anion Recognition Sites. Efficient Fluoride Anion Receptors, *J. Am. Chem. Soc.*, **2002**, 124, 1134-1135.
- Endres, G. F., J. Epstein, Synthesis of Some Hydroxamic acids. Reactivity with Isopropyl Methylphosphono fluoridate (GB), *J. Chem. Soc.*, **1959**, 24, 1497-1501.