Polymer Synthesis and Characterization of a Molecularly Imprinted Sorbent Assay for Atrazine

Keywords: *Molecular imprinting; atrazine; polymer; residue analysis*

INTRODUCTION

Molecular imprinting technology utilizes functionalized polymers formed in the presence of a "print" molecule for developing binding assays for the print molecule itself or a structurally similar one (Wulff, 1984, 1986). The synthesis of molecular imprints involves several steps as outlined by Sellergen and Andersson (1990). First, functionalized monomers are mixed with the print molecule to which they bind covalently and/or noncovalently. Next, this "print assembly" is copolymerized with excess cross-linking agent, forming a rigid polymer. The print molecule is then extracted or hydrolyzed from the polymer. It is hypothesized that molecular imprint recognition of the print molecule is attributed to the formation of functional groups in a particular spatial arrangement within the polymer matrix conforming to that of the print molecule (Wulff and Schauhoff, 1991). In addition, shape-selective cavities may also contribute to binding, particularly with nonfunctional print molecules such as aromatic hydrocarbons (Dunkin et al., 1993).

A commonly used polymer matrix uses the functional monomer methacrylic acid (MAA) with ethylene glycol dimethylacrylate (EGDMA) as the cross-linking monomer [eg., O'Shannessey et al. (1989), Andersson et al. (1990), and Vlatakis et al. (1993)]. However, other matrices have been used. For example, weakly basic functional monomers have been used for binding oxygen-containing print molecules (Ramstrom et al., 1993), while monomers with aromatic functionalities have been used for binding planar aromatics (Dunkin et al., 1993).

The properties of molecular imprints have often been studied using chromatographic systems such as high-performance liquid chromatography (HPLC) and thin-layer chromatography (TLC), in which the polymer is used as the solid phase. This approach was applied to the separation of racemic mixtures of sugars (Wulff and Schauhoff, 1991) and amino acid derivatives (Sellergren et al., 1985; Matsiu et al., 1993; Kriz et al., 1994). Receptor binding assays were used to study molecular imprints (Sellergren and Andersson, 1990; Wulff and Schauhoff, 1991) and resulted in the development of the molecularly imprinted sorbent assay (MIA) (Vlatakis et al., 1993). This MIA was a radioassay that used a molecularly imprinted polymer as the receptor in a competition binding assay. The MIA was as sensitive as a commercially available immunoassay for the two drugs of interest (theophylline and diazepam).

Molecular imprinting technology is less expensive than antibody production and may offer an alternative in situations when the cost of antibody production is prohibitive or antibody performance is a problem. In addition, molecular imprint polymers are highly resistant to organic solvent effects, unlike antibodies (or other biological receptors). Thus, molecular imprints may have applications for the analysis of highly lipophilic compounds (e.g., PCBs or dioxins) either in a sample cleanup step or in a detection method. The overall purpose of this research is to evaluate molecular imprinting techniques for the analysis of analytes of agricultural or environmental importance.

The *s*-triazine herbicide atrazine (see Figure 1) was chosen as a model system for study. It is a nitrogenous heterocycle possessing two secondary amino groups and has been shown to form complexes with acetic acid in organic solvent (Wellhouse and Bleam, 1993). Therefore, atrazine should bind to the functional monomer MAA under the same conditions (i.e., in an organic solvent). The *s*-triazines are good models for these studies because they are relatively inexpensive, nontoxic, and stable. Thus, the gram quantities necessary in a typical reaction can be handled without extraordinary precautions. In addition, immunoassay and HPLC methods are currently available for comparative purposes, and a library of other *s*-triazines is available for specificity characterization of an MIA.

MATERIALS AND METHODS

Chemicals. Atrazine and the other *s*-triazines (>97% purity) used were gifts from Ciba-Geigy (Greensboro, NC) except [*ring*-UL-^{14}C]atrazine (25 mCi/mmol), which was purchased from Sigma (St. Louis, MO). Methacrylic acid (MAA, functional monomer) and ethylene glycol dimethylacrylate (EGDMA, comonomer) were purchased from Aldrich (Milwaukee, WI). 2,2-Azobis(isobutryonitrile) (AIBN, initiator) was purchased from Chem Service (West Chester, PA). Omnisolv chloroform was from EM Science (Gibbstown, NJ), and HPLC grade acetonitrile and phosphoric acid (85% v/v) were from Fisher Scientific (Fair Lawn, NJ). High-purity water was obtained from a MILLI-RO4 purification system (Millipore Corp., Bedford, MA). Liquid scintillation cocktail was Ready Safe from Beckman (Fullerton, CA).

Equipment. Minisieves were purchased from Whatman (Hillsboro, OR). Liquid scintillation counting (LSC) was performed on a Model 1212 Rackbeta liquid scintillation counter from LKB-Wallac (Turku, Finland). High-performance liquid chromatography was performed using a Dionex (Sunnyvale, CA) microbore system consisting of an advanced gradient pump and a VDM-2 variable-wavelength detector monitored at 225 nm and controlled using an AI-450 chromatography workstation. The column was a 15 cm × 2.1 mm, 5 μm, Supelcosil LC-18 from Supelco (Bellefonte, PA). The isocratic solvent system was 50% acetonitrile (solvent A) in phosphoric acid (0.1% v/v) (solvent B). The gradient solvent system was 10% solvent A in solvent B, which was maintained for 1 min postinjection. Then a gradient was initiated resulting in 80% solvent A in B at 5 min. This was maintained until 10 min postinjection. The initial solvent condition was again attained at 15 min. The flow rate was 0.25 mL/min. Samples (25 μL) were injected onto the system using a Spectra-Physics (San Jose, CA) SP 8880 autosampler.

Data calculations utilized Excel spreadsheet software (Microsoft Corp., Redmond, WA) and SOFTmax 2.01 software (Molecular Devices Corp., Menlo Park, CA).

Polymer Synthesis. The procedure was an adaptation of the method used by Vlatakis et al. (1993) for the synthesis of anti-theophylline polymer. Atrazine (the print molecule) (1.128 g, 5.22 mmol) was added to 50 mL of chloroform in a 250 mL Erlenmeyer flask. To this solution [50 mL of chloroform containing atrazine (specific polymer) or 50 mL of chloroform without print molecule (control polymer)] were added MAA (1.8 g, 20.9 mmol) and EGDMA (18.7 g, 94.3 mmol). This was followed by 0.24 g of AIBN (1.76 mmol). The flask was capped with a rubber septum and vented with a syringe needle. The reaction mixture was sparged with helium for 5 min while in a sonicating water bath and heated to 60 °C on a water bath.

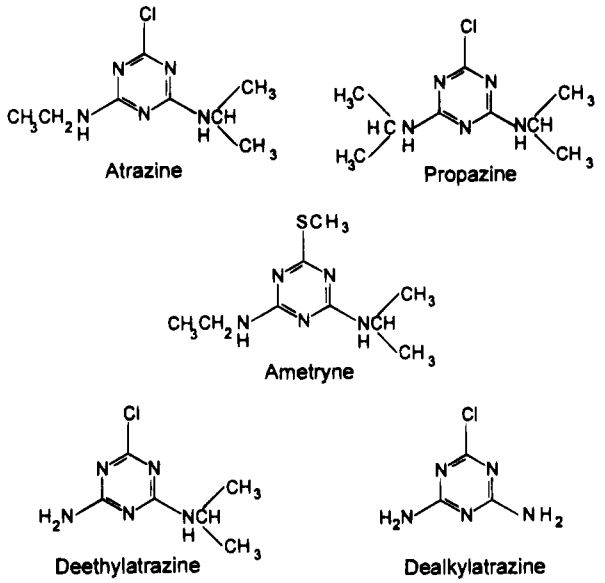

Figure 1. Various *s*-triazines used in this study.

After 30 min, a white, glassy polymer began to form in the flask. The reaction was carried out for 23 h, at which time the solvent had evaporated. The flask was set in a vacuum oven at 60 °C for a minimum of 15 h. The polymer was removed from the flask and ground by hand with a mortar and pestle. The powder was sieved with water through a 25 μm sieve and recovered by filtration on No. 1 Whatman filter paper. The powder (<25 μm) was sedimented several times in acetonitrile (5 × 50 mL) to remove the fine material. The coarse polymer was extracted with chloroform (5 × 30 mL) to remove residual print molecule, vacuum oven-dried at 60 °C, and stored in a capped vial at room temperature. The filtrates from wet-sieving (water), sedimentation (acetonitrile), and extraction (chloroform) were analyzed for the presence of atrazine by HPLC. The procedure was repeated for the synthesis of both the specific and the control polymers.

Determination of Polymer Recognition of Atrazine by Radioassay. Specific or control polymer (0, 10, 20, 40, 80, or 160 mg) was added to 1.8 mL polypropylene centrifuge tubes. One milliliter of acetonitrile containing 0.14 μCi of [^{14}C]atrazine (1.2 μg) was added to each tube. The tubes were vortexed to suspend the polymer and incubated for 23 h at room temperature without agitation. The tubes were centrifuged at 1000*g* for 10 min. Duplicate aliquots (20−200 μL) of the supernatants were mixed with liquid scintillation cocktail and analyzed by LSC.

Titration of [^{14}C]Atrazine on the Specific and Control Polymers. Either specific or control polymer (50 mg) was added to each tube. A series of [^{14}C]atrazine standards were made in acetonitrile ranging from 0.10 to 0.0005 μCi/mL (800−0.004 μg/mL). One milliliter of each standard was added to a tube containing either specific or control polymer. The tubes were vortexed and incubated for 23 h at room temperature with agitation. Following centrifugation, duplicate aliquots (200 μL) of the supernatants were analyzed by LSC.

Competitive Inhibition Molecular Imprint Assay for Atrazine. Standards of atrazine, propazine, ametryne, deethylatrazine, and chlorodiamino-*s*-triazine (Figure 1) were made in acetonitrile ranging from 1000 to 0.1 μg/mL. [^{14}C]Atrazine was added to each standard as well as acetonitrile containing no competitor to give 4 nCi/mL. One milliliter of each solution was added to each tube containing 50 mg of polymer (either specific or control). The tubes were vortexed and incubated for 23 h at room temperature with agitation. Following centrifugation, duplicate aliquots (200 μL) of the supernatants were analyzed by LSC. The percent dpm added which was bound to the polymer was calculated as a residual from the amount measured in the supernatant according to the equation

$$\% \text{ bound} = [(\text{total dpm added} - \text{dpm in supernatant})/\text{total dpm added}] \times 100 \quad (1)$$

Percent inhibition of the no-competitor control (% inhibition) was calculated as

$$\% \text{ inhibition} = [1 - (\% \text{ bound}_x - \% \text{ bound}_c)/(\% \text{ bound}_s - \% \text{ bound}_c)] \times 100 \quad (2)$$

where x, c, and s are the sample, control polymer (no competitor), and specific polymer (no competitor). IC_{50} values (concentration of inhibitor that produces a 50% decrease in signal of the no-competitor control) for the various *s*-triazine analogs were derived from the four-parameter curve fitting function in SOFTmax. Percent reactivity for each analog was calculated as follows:

$$\% \text{ reactivity} = (IC_{50} \text{ atrazine}/IC_{50} \text{ analog}) \times 100 \quad (3)$$

Competitive Enzyme-Linked Immunosorbent Assay (cELISA). The cELISA was performed using monoclonal antibody AM7B2 as described in Muldoon and Nelson (1994). The monoclonal antibody was a gift from A. E. Karu, Hybridoma Facility, University of California, Berkeley, CA, and is described in detail elsewhere (Karu et al., 1991). IC_{50} values and percent reactivities for the analogs were calculated as described above.

RESULTS AND DISCUSSION

Polymer Synthesis. Polymers were made in the presence of atrazine (specific polymer) and without atrazine present (control polymer). The filtrates from wet-sieving (water), from sedimentation (acetonitrile), and from extraction (chloroform) were analyzed by HPLC for atrazine. In the first synthesis, atrazine (162 μg) was detected in the initial 130 mL of the chloroform extract. This amounted to a recovery of 40 ng of atrazine/mg of polymer. Atrazine was not detected in the wet-sieving or acetonitrile fractions. In the second synthesis, atrazine was detected in the water (0.5 mg) and in the acetonitrile fractions (1 mg), giving a recovery of 221 ng/mg of polymer. Further extraction of an aliqout (50 mg) of the specific polymer with 90% methanol in acetic acid (1.0 mL) was negative for atrazine. The small amount of atrazine extracted (less than 0.5%) suggests that only a small amount of atrazine is available for extraction, that the atrazine was very tightly bound in the polymer matrix, or that it degraded in the polymerization process. It is difficult to measure the amount of surface area available for extraction, but clearly much of the polymer will not be available for extraction. Wellhouse and Bleam (1993) showed that the strong hydrogen bonding of atrazine to acetic acid is cooperative; both partners in the complex accept and donate protons. Perhaps atrazine in these complexes was not dissociated and only more weakly bound atrazine was removed during the extraction process. In addition, atrazine is susceptible to degradation via free radical reactions (Plimmer et al., 1971; Esser et al., 1975), which also describe the mechanism of polymer formation.

Analysis of Molecular Imprints for Atrazine Binding. Figure 2 shows the results from direct binding experiments of [^{14}C]atrazine to the atrazine-specific and the control polymers. The specific polymer bound more atrazine than the control polymer at all polymer levels tested. The amounts of atrazine bound

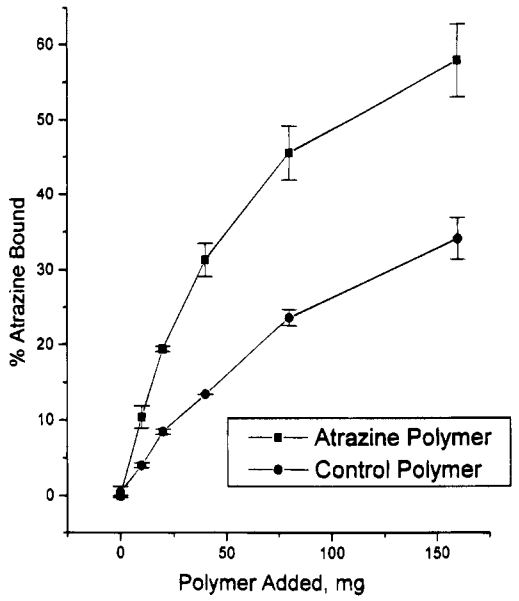

Figure 2. Atrazine binding to various amounts of atrazine-specific and control polymer. Error bars represent the standard deviation from two experiments that used different batches of polymer.

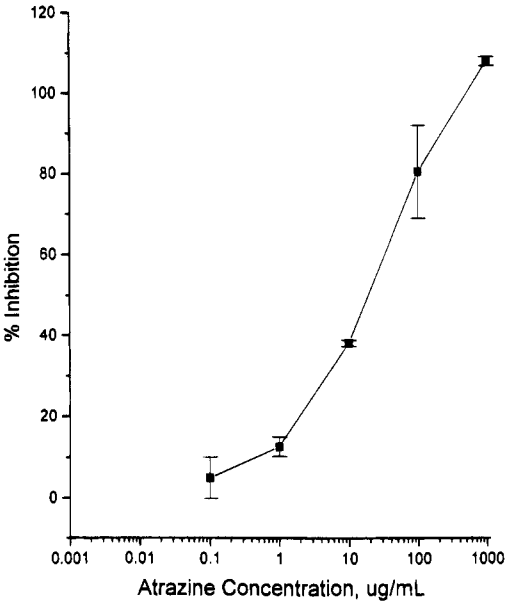

Figure 3. Typical MIA for atrazine. The assay was performed in acetonitrile using [^{14}C]atrazine as tracer. Error bars represent the standard deviation from triplicate experiments.

per milligram of polymer for 10, 20, 40, 80, and 160 mg of specific polymer were 10.1, 9.6, 7.7, 5.6, and 3.6 ng/mg, respectively. This nonlinearity is probably due to differences in equilibration times depending on the amount of polymer added. The use of a finer particle size polymer would result in an increased amount of atrazine bound per milligram of polymer due to an increase in available surface area for binding; however, this was not used due to difficulties in handling. The high reproducibility for the synthesis of polymers possessing similar binding characterisitics is shown by the relatively small error bars associated with duplicate syntheses. These data clearly show, however, that it was possible to synthesize a molecular imprint which binds atrazine (the print molecule) significantly more than did a control polymer.

Competitive Inhibition Molecularly Imprinted Sorbent Assay (cMIA). From the previous experiments it was decided that 50 mg of polymer per assay be used for MIA development since a relatively high atrazine binding rate is still maintained (approximately 7 ng of atrazine/mg of polymer) and the polymer can be easily weighed out with minimum measurement error. Therefore, [^{14}C]atrazine (800−0.004 μg) was titrated on 50 mg of polymer to determine the appropriate amount for use in an MIA. The specific polymer bound approximately 30% of the [^{14}C]atrazine added, and this was consistent throughout the entire range studied (data not shown). In contrast, the control polymer bound only approximately 15% of the [^{14}C]atrazine except at 800 μg, when binding decreased to 10%. The decrease in binding in the 800 μg sample may indicate saturation of nonspecific binding sites in the control polymer. No such saturation effect was observed with the specific polymer. Therefore, we used 4 nCi (33 ng) of atrazine per assay for the MIA since this gave an adequate signal in the supernatant for the specific polymer with no competitor present (approximately 1000 dpm/200 μL).

A typical competitive inhibition curve for the atrazine MIA is shown in Figure 3. The assay appears log-linear in the range of 1.0−100 μg/mL (4.6−462 μM) atrazine. Above this range the atrazine MIA was susceptible to nonspecific inhibition effects. The analyte sensitivity reported here was very similar to that reported by Vlatakis et al. (1993) for the nitrogenous heterocyclic analytes theophylline (14−224 μM) and diazepam (0.44−28 μM). The use of a tracer with higher specific activity should improve the sensitivity of the atrazine MIA.

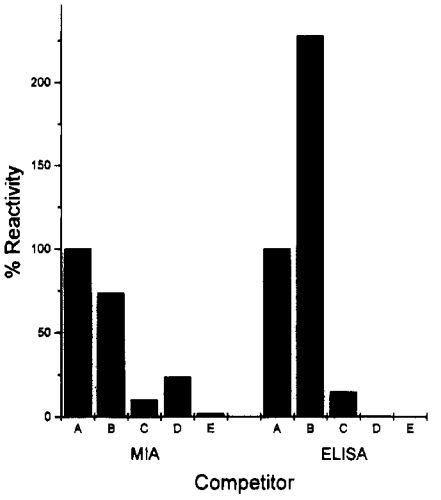

Figure 4. *s*-Triazine reactivity profiles for the atrazine MIA and the atrazine ELISA utilizing monoclonal antibody AM7B2: A, atrazine; B, propazine; C, ametryne; D, deethylatrazine; E, didealkylated atrazine. The percent reactivity was determined by comparison of the amount of competitor that produced a 50% inhibition of either [^{14}C]atrazine binding to specific polymer (MIA) or the absorbance value of the no-competitor control (ELISA).

Selectivity Characterization of the Atrazine MIA. The atrazine MIA was characterized for reactivity toward selected *s*-triazines (Figure 1) which differed from atrazine in either chloro or *N*-alkyl substitution or N-dealkylation. Reactitivity results are summarized in Figure 4. Also shown in Figure 4 is the reactivity pattern observed for the *s*-triazine monoclonal antibody AM7B2 analyzed for binding to the same compounds using a competition ELISA (Karu et al., 1991; Muldoon and Nelson, 1994). The atrazine MIA recognized propazine nearly to the same extent as atrazine. Thus, small changes in alkyl side chain structure had little

effect on recognition. However, analyte recognition decreased as a function of substitution of the chlorine atom with a thiomethyl group as well as N-dealkylation.

The recognition pattern observed for the atrazine ELISA was similiar to that seen with the MIA. There was a decrease in antibody recognition as a function of chlorine substitution or N-dealkylation. However, the monoclonal antibody showed very strong recognition of propazine. This indicated that the antibody recognized the *N*-isopropyl group better than it did the *N*-ethyl group. This was not observed with the atrazine MIA. Vlatakis et al. (1993) also reported similarities between the recognition patterns for various MIAs and analogous immunoassays. These results may suggest that analyte recognition in these systems may occur through similar mechanisms. It should be possible to produce molecular imprints with differing reactivities toward individual members within a chemical class (such as the *s*-triazines) by varying the imprint molecule as well as the functional monomer(s) used for synthesis.

Conclusions. We have been able to synthesize a molecular imprint for atrazine. Imprint characterisitics were highly reproducible. The imprints were used in an MIA for atrazine which showed selectivity similar to that of a commonly used antibody for atrazine. The sensitivity of the assay for atrazine was similar to that for other analytes for which MIAs have been developed. However, the atrazine MIA is less sensitive than analogous immunoassays. The MIA, unlike the immunoassay, was performed in an organic solvent. This feature would allow for the analysis of crude organic solvent sample extracts and alleviate the need for solvent exchange into aqueous-based systems, which is normally required for immunoassays.

These data indicate that the technique is a reproducible method that should be generally applicable to compounds that possess recognition groups to which functionalized polymers may bind. Given the various functional chemistries available for synthesis, the technique should be applicable to a large number of environmentally and agriculturally important compounds, both as a detector in MIAs and as a chromatography or sample preparation matrix. We are currently investigating the use of this technology for developing nonradioactive MIAs and selective chromatography matrices.

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