Atrazine transforming polymer prepared by molecular imprinting with post-imprinting process†

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Molecularly imprinted polymers bearing atrazine transforming activity were prepared by using newly designed templates that are atrazine analogues attached with an allyl or a styryl group *via* a disulfide bond at the 6-position, methacrylic acid as a functional monomer and styrene/divinylbenzene as crosslinkers. After polymerization, the disulfide bond was reduced to remove the atrazine moiety from the polymer matrix, followed by oxidation of the remaining thiol group to generate sulfonic acid (post-imprinting treatment), so that both a methacrylic acid residue and a sulfonic acid residue existed in an atrazine-imprinted cavity. The polymers indicated the selective binding of triazine herbicides and catalytic activity for methanolysis at the 6-position of atrazine, yielding a low toxic atraton.

Introduction

Molecular imprinting has come to be regarded as one of the potentially promising and convenient methods for the preparation of synthetic polymers having specific binding/catalytic sites.[1] In this technique, a molecular template that is often a target molecule itself or its analogue is added to a prepolymerization mixture containing crosslinker(s) and other monomers. After polymerization, the template molecule is removed from the obtained polymer, yielding three dimensional binding cavities specific for the target molecule.

Many molecularly imprinted catalysts (MIC) for various reactions have been prepared by using various template molecules such as transition state analogues, substrates and products.[2] For the preparation of MIC, both covalent[3] and non-covalent[4] molecular imprinting have been used, in which the most important issue is how we can insert catalytic functional groups into the specific binding sites, since randomly located catalysts may cause non-specific catalytic reactions.

Covalent molecular imprinting, in which polymerizable templates are used, can provide fairly homogeneous binding sites, because the remaining functional groups in the resulting polymers are only located inside the binding sites after the cleavage of templates. Therefore covalent molecular imprinting would be more favorable for the introduction of desirable functional groups into target-selective binding sites than non-covalent techniques in order to develop catalytic activities.

Herein we propose a technique for preparing substrate-selective catalysts by the combined use of covalent and non-covalent molecular imprinting coupled with a post-imprinting chemical modification (post-imprinting process). As a model catalytic reaction, methanolysis assisted by acids of a herbicide, atrazine (6-chloro-N^2-ethyl-N^4-isopropyl-1,3,5-triazine-2,4-diamine), was employed, where 6-Cl is replaced by OCH_3 to yield atraton (6-methoxy-N^2-ethyl-N^4-isopropyl-1,3,5-triazine-2,4-diamine).

Atrazine has two amino groups and the three nitrogen atoms of a triazine in its structure and they can be utilized to form hydrogen bonding with carboxylic acid. By using hydrogen bonding, atrazine-imprinted polymers have been successfully prepared, in which specific binding was shown for atrazine.[5] Thus we utilized this non-covalent atrazine imprinting system as a way to construct carboxylic acid-based binding cavities for the recognition of atrazine. As a catalytic functional group inside the recognition cavities, we intended to introduce a strongly acidic functional group such as sulfonic acid at a desirable position inside the specific binding sites, preferably around the 6-Cl position of atrazine in its bound position, because the nucleophilic reaction should proceed at the 6-position of atrazine.

For this purpose, novel atrazine analogue template molecules attached to a polymerizable group *via* a disulfide bond at the 6-position were designed. By using these templates, molecularly imprinted polymers bearing an atrazine transforming activity were prepared, involving covalent and non-covalent molecular imprinting (Scheme 1). This strategy can introduce minimum amounts of sulfonic acid at appropriate positions and this may lead to enhanced specificity due to low non-specific binding.

Results and discussion

Firstly, we designed Template 1 (T1: allyl 4-ethylamino-6-isopropylamino-1,3,5-triazine-2-yl disulfide) that is an atrazine analogue attached to an allyl group *via* a disulfide bond at the 6-position, in order to obtain MIC bearing carboxylic acid-based atrazine imprinted cavities with a sulfonic acid group located inside the cavity. The imprinted polymers were prepared by radical polymerization of a monomer mixture containing T1, methacrylic acid (MAA) as a functional monomer for the construction of atrazine binding sites, and divinylbenzene and styrene as crosslinkers in chloroform. After polymerization, the disulfide bonds originating from T1 were reduced by $NaBH_4$ to remove the atrazine moiety, yielding the atrazine binding cavities. In the final step, the remaining thiol groups in the polymers were transformed

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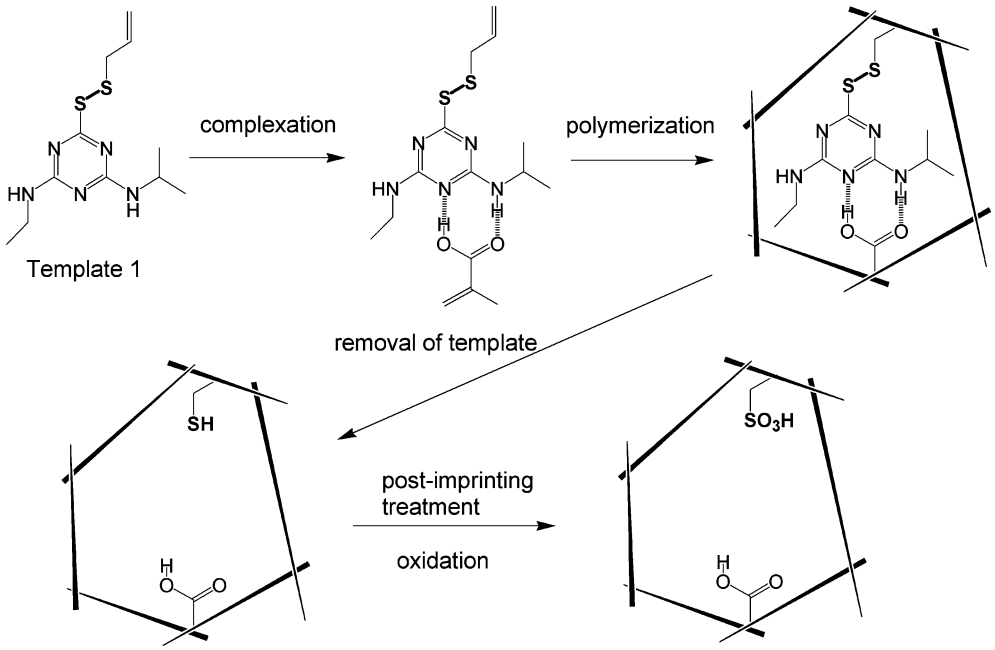
† Electronic supplementary information (ESI) available: Michaelis–Menten plots of IP(T1) and IP(T2), Lineweaver–Burk plot of IP(T2), selectivity of catalysis by IP(T2). See DOI: 10.1039/b612407k



Scheme 1 Illustration of the preparation of atrazine transforming polymers using the post-imprinting process.

into sulfonic acids by oxidation with hydrogen peroxide (post-imprinting process).[6] After all the processes were completed, the imprinted polymer IP(T1) had binding/catalytic sites containing both MAA and sulfonic acid residues. The conversion of T1 in the obtained polymer was about 20% (215 μmol per g polymer) as estimated by the S-content determined by elemental analysis.[7]

Binding properties of IP(T1) were investigated by batch binding tests, where the imprinted polymers were incubated with triazine herbicides and other pesticides (100 μM) in chloroform for 24 h at 25 °C. Under the conditions, no catalytic reaction proceeded because no nucleophilic reagent existed. IP(T1) showed strong adsorption for triazine herbicides (atrazine, atraton, ametryn, and cyanazine), while other pesticides (thiuram, propyzamide, and iprodion) were hardly adsorbed (Fig. 1). These results clearly indicate that atrazine imprinting was successfully achieved in IP(T1). The binding of cyanazine, that has a bulky substituent, was weaker than others, suggesting that the polymers cannot recognize the difference between Cl and OCH_3 substitutes at the 6-position, however they can recognize larger side chains even if the base structure is the same.

The transformation of atrazine (methanolysis of atrazine to atraton) by IP(T1) was examined by incubation with atrazine in $CHCl_3$–MeOH (9 : 1, v/v) at 40 °C. Transformation velocities of atrazine in the presence of IP(T1) were estimated by monitoring the atraton production with time. Upon the investigation of the effects of atrazine concentrations on the reaction velocities, saturation behaviors were observed and a linear Lineweaver–Burk plot was obtained (Fig. 2), suggesting that the reactions may proceed with Michaelis–Menten kinetics under the conditions employed. Therefore, similar to enzymatic catalysis, the substrate bound to the binding site in the imprinted polymers should react with a nucleophilic reagent, methanol. Kinetic parameters were calculated to be as follows; V_{max}: 2.1×10^{-7} M min^{-1}, K_m: 1.6×10^{-4} M, k_{cat}: 4.9×10^{-4} min^{-1}, whereas the total concentration of catalytic sites was estimated from the result of elemental

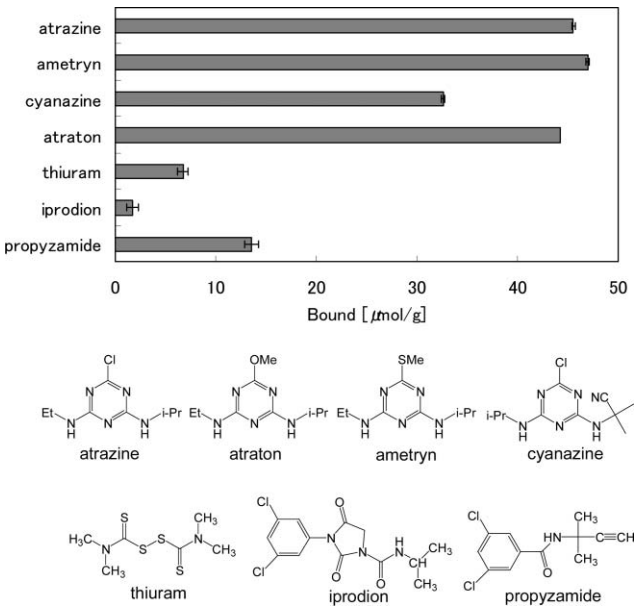


Fig. 1 Binding selectivity of IP(T1). Selectivity is shown by binding amounts for 100 μM substrate solution.

analysis (4.3×10^{-4} M). At high substrate concentrations, product inhibition may occur. To avoid this, other nucleophilic reagents could be helpful if chemical properties of products can be significantly changed after the nucleophilic reaction.

To prove that the catalytic reaction proceeded inside the binding sites of the imprinted polymers, competition experiments were carried out using an inactive triazine herbicide, ametryn, for methanolysis with two different atrazine concentrations. The reaction velocities diminished with the increase of ametryn concentrations. This means that atrazine is transformed in the binding sites that can be bound by ametryn, *i.e.* the catalytic site is

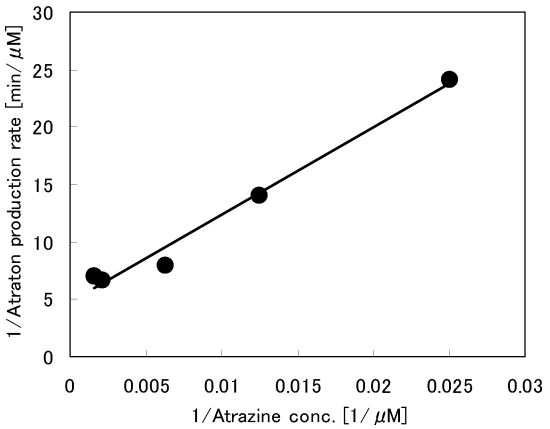


Fig. 2 Lineweaver–Burk plots on atraton production catalyzed by IP(T1) at 40° C; $V_{max} = 2.1 \times 10^{-7}$ M min^{-1}, $K_m = 1.6 \times 10^{-4}$ M.

located inside the cavity constructed during the imprinting process. The inhibition constant K_i was estimated to be 2.6×10^{-6} M from a Dixon plot (Fig. 3). The figure is smaller than for the previously reported MIC prepared by non-covalent molecular imprinting using 2-sulfoethyl methacrylate as a catalytic monomer (4.7×10^{-5}M),[8] indicating that the homology of binding/catalytic sites could be improved by the proposed combined method involving covalent and non-covalent imprinting.

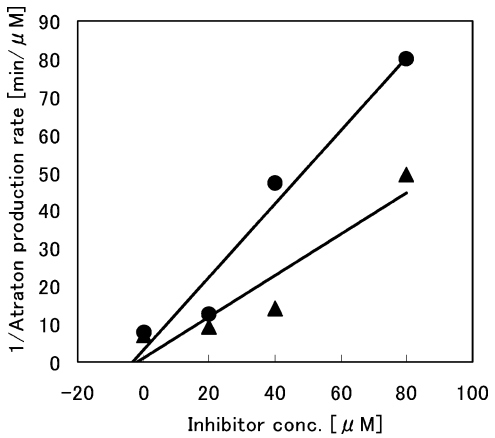


Fig. 3 Inhibition of the transformation activity by ametryn. Atrazine (▲: 160 μM, ●: 320 μM) and ametryn were incubated with IP(T1).

The specificity of the catalytic activity for various triazine herbicides was evaluated by the ratio of the amount of product to the initial amount of substrate after 12 h (Fig. 4). IP(T1) exhibited catalytic activities for all the triazine substrates and atrazine selectivity was observed. This also proved that the atrazine-selective binding sites catalyze the transformation of triazine herbicides.

Because the co-polymerization efficiency of T1 with other monomers was not good (20%), we designed Template 2 (T2: 4-vinylbenzyl (4-ethylamino-6-isopropylamino-1,3,5-triazine-2-yl disulfide). It has a styryl group instead of an allyl group that should easily co-polymerize with styrene/divinylbenzene. For the preparation, a smaller amount of T2 was used because T2 may give a high conversion rate and further, higher content of crosslinker would give more highly selective binding sites. As expected, when an imprinted polymer was prepared using T2 (IP(T2)), the

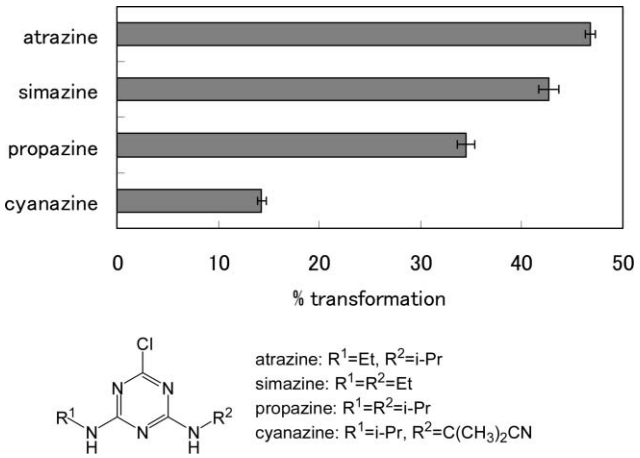
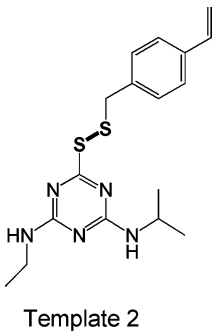


Fig. 4 Proportions of transformed substrates catalyzed by IP(T1) for 250 μM substrate solutions after 12 h incubation at 40 °C.

conversion rate was almost 100% (256 μmol per g polymer), as estimated by the S-content in elemental analysis,[9] and the binding specificity observed was higher than that of IP(T1) (Fig. 5).



Template 2

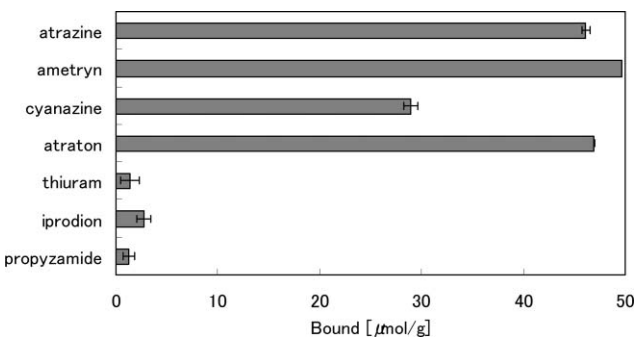


Fig. 5 Binding selectivity of IP(T2). Selectivity is shown by binding amounts for 100 μM substrate solutions.

Table 1 summarizes kinetic parameters of IP(T1) and IP(T2) (detailed data are given in the ESI†). The value of V_{max} for IP(T2) was higher than that for IP(T1), meaning that more catalytic sites exist per unit weight of IP(T2) than in IP(T1). Methanolysis of atrazine was catalyzed by the sulfonic acid residues in the binding sites and with the concentration of catalytic sites estimated by the S-contents in the polymers, k_{cat} values of IP(T1) and IP(T2) were calculated to be 4.9×10^{-4} and 1.2×10^{-3} min^{-1}, respectively; this is a measure of the catalytic activity. These results reveal that the imprinting process using T2 can provide higher catalytic ability,

Table 1 Kinetic parameters of the imprinted polymers for the catalytic atrazine transformation

	IP(T1)	IP(T2)
V_{max} (10^{-7} M min^{-1})	2.1	6.0
K_m (10^{-4} M)	1.6	5.7
k_{cat} (10^{-4} min^{-1})	4.9	12

IP(T1): $[E]_{total} = 4.3 \times 10^{-4}$ M; IP(T2): $[E]_{total} = 5.1 \times 10^{-4}$ M.

i.e. the population of properly working sites may be higher. The K_m of IP(T1) was smaller than that of IP(T2). Thus, relatively more stable substrate–catalyst complexes may form in the less sterically hindered binding sites of IP(T1) because of the absence of a phenylene group. Since the catalytic activity was affected by the conversion rate of the templates used and the steric hindrance of the linkers between the disulfide and vinyl groups, more careful design of the template molecules would lead to the development of higher catalytic activity.

Conclusions

We proposed new MIC preparation methods involving the combination of covalent and non-covalent molecular imprinting processes with post-imprinting chemical modification for the introduction of one catalytic site per one specific binding cavity. The resulting imprinted polymer has a catalytic activity for the transformation of atrazine to atraton with Michaelis–Menten kinetics-like behavior. This method will provide a new way to introduce catalytic functional groups into molecularly imprinted cavities, resulting in more homogeneous catalytic sites.

Experimental

Preparation of T1 and T2

4-Ethylamino-6-isopropylamino-2-(4-methoxybenzylsulfanyl)-1,3,5-triazine (1). A solution of atrazine (2.15 g, 10 mmol), 4-methoxy-α-toluenthiol (1.4 ml, 10 mmol), sodium-*tert*-butoxide (1.92 g, 20 mmol), tetrakis (triphenylphosphine)palladium(0) (1.15 g, 1.0 mmol) in DMF (200 ml) was stirred at 100 °C for 20 h under nitrogen. The resulting mixture was concentrated and extracted with $CHCl_3$. The mixture was purified by silica gel column chromatography (dichloromethane–methanol = 100 : 1) to yield **1** (2.26 g, 68%); δ_H (300 MHz; $CDCl_3$; Me_4Si) 1.23 (9H, m, CH_3), 3.45 (2H, m, NCH_2), 3.80 (3H, s, OCH_3), 4.38 (3H, m, NCH and SCH_2), 6.78 (2H, d, ArH), 7.21 (2H, s, NH) and 7.20 (2H, d, Ar–H).

4-Ethylamino-6-isopropylamino-1,3,5-triazine-2-thiol (2). A solution of **1** (1.97 g, 5.9 mmol) in TFA (20 ml) was stirred at room temperature for 6 h. The resulting mixture was diluted with $CHCl_3$ and was washed with $NaHCO_3$ solution. The crude product was purified by silica gel column chromatography (chloroform–ethyl acetate = 1 : 1) to yield **2** (1.29 g, 100%); δ_H (300 MHz; $CDCl_3$; Me_4Si) 1.25 (9H, m, CH_3), 3.48 (2H, m, NCH_2), 4.20 (1H, m, NCH), 8.70 (1H, s, NH) and 8.99 (1H, s, NH).

Allyl 4-ethylamino-6-isopropylamino-1,3,5-triazin-2-yl disulfide (T1). A solution of **2** (422 mg, 1.98 mmol) and *N*-allylsulfanyl succinimide (810 mg, 1.98 mmol) in benzene (45 ml) was stirred at 70 °C for 12 h. Afterwards hexane was added to the mixture, which was filtered to extract precipitate and the filtrate was evaporated. The crude product was purified by silica gel column chromatography (dichloromethane–methanol = 200 : 1) to yield T1 (215 mg, 44%); δ_H (300 MHz; $CDCl_3$; Me_4Si) 1.25 (9H, m, CH_3), 3.49 (4H, m, NCH_2 and SCH_2), 5.15 (2H, m, $=CH_2$) and 5.90 (1H, m, =CH).

4-Vinylbenzyl 4-ethylamino-6-isopropylamino-1,3,5-triazin-2-yl disulfide (T2). A solution of **2** (296 mg, 1.40 mmol), di(4-vinylbenzyl)disulfide (2.70 g, 11.2 mmol) and triethylamine (3.5 ml) in benzene (150 ml) was stirred at 80 °C for 48 h. The crude product was purified by silica gel column chromatography (dichloromethane–ethyl acetate = 10 : 1) to yield Template 2 (150 mg, 30%); δ_H (300 MHz; $CDCl_3$; Me_4Si) 1.23 (9H, m, CH_3), 3.45 (2H, m, NCH_2), 4.15 (3H, m, NCH and SCH_2), 5.19 and 5.70 (2H, d, $=CH_2$), 6.70 (1H, m, =CH) and 7.33 (4H, m, ArH).

Preparation of IP(T1) and IP(T2)

IP(T1). T1 (720 mg, 2.52 mmol), the functional monomer MAA (214 μl, 2.52 mmol), divinylbenzene (1.08 μl, 7.56 mmol) and styrene (533 μl, 3.78 mmol) as crosslinkers were mixed in 1.64 μl of chloroform. Polymerization was initiated by photo-irradiation using a radical initiator, 2,2′-azobis(isobutyronitrile) (60 mg) for 12 h at 5 °C, and the solution was heated for 4 h at 60 °C to remove the solvent. The resulting polymer was ground to obtain polymer particles. The polymer particles (2.0 g) and sodium borohydride (2.0 g, 20 eq. with respect to the disulfide bond) were suspended in methanol (80 ml). The mixture was stirred. This cleavage procedure was repeated a further two times. After reduction, the polymer particles were washed with methanol–water (9 : 1) and methanol at room temperature. Post-imprinting treatment to convert of thiol groups in the polymers to sulfonic acid groups was performed as follows. The polymer particles were suspended in H_2O_2–acetic acid (80 ml, 1 : 1, v/v) and stirred at room temperature for 12 h. This post-imprinting treatment was repeated a further two times. After post-imprinting treatment, the polymer particles were stirred in 100 mM sulfuric acid–methanol (1 : 1) for 8 h at room temperature, then were washed with methanol.

IP(T2). T2 (181 mg, 0.50 mmol), the functional monomer MAA (42.5 μl, 0.50 mmol), divinylbenzene (1.67 ml, 10 mmol) and styrene (573 μl, 5.0 mmol) as crosslinkers were mixed in 1.6 ml of chloroform. Polymerization was initiated by photo-irradiation using a radical initiator, 2,2′-azobis(isobutyronitrile) (59 mg) for 20 h at 5 °C, and the solution was heated for 4 h at 60 °C to remove the solvent. The resulting polymer was ground to obtain polymer particles. The polymer particles (1.34 g) and sodium borohydride (380 mg, 20 eq. with respect to the disulfide bond) were suspended in methanol (100 ml). The mixture was stirred. This cleavage procedure was repeated a further three times. After reduction, the polymer particles were washed with methanol–water (9 : 1) and methanol at room temperature. Post-imprinting treatment to convert the thiol groups in the polymers to sulfonic acid groups was performed as follows. The polymer particles were suspended in H_2O_2–acetic acid (100 ml, 1 : 1, v/v) and stirred at room temperature for 8 h. This post-imprinting treatment was repeated

a further two times. After post-imprinting treatment, the polymer particles were stirred in 1.0 N hydrochloric acid–methanol (1 : 1) for 12 h at room temperature, then were washed with methanol.

Binding selectivity of imprinted polymers

The polymer particles (3.0 mg) were incubated with atrazine, ametryn, cyanazine, atraton, thiuram, iprodion or propyzamide (100 μM at 25 °C) in chloroform (1.5 ml) for 24 h. After incubation, the suspensions were filtered, and the filtrates (1.0 ml) were dried *in vacuo.* The residues were dissolved in acetonitrile (1.0 ml) and analyzed by an HPLC system consisting of a 306 pump, a 234 autoinjector (Gilson), a 170 Diode Array Detector (Agilent) and a 502 degasser (M & S Instruments) with a reversed phase column (TSKgel ODS-80TsQA) and an eluent of acetonitrile–10 mM ammonium acetate buffer (55 : 45 v/v, pH 6.0, 1.0 ml min^{-1}).

Kinetic experiments

The polymer particles (3.0 mg) were incubated with atrazine (0–400 μM at 40 °C) in methanol–chloroform (1.5 ml, 1 : 9, v/v) for 40, 80, 120, 160 and 200 min. After incubation, the suspensions were filtered, and the filtrates (1.0 ml) were dried *in vacuo.* The residues were dissolved in acetonitrile (1.0 ml) and analyzed by HPLC with a reversed phase column (TSKgel ODS-80TsQA) and an eluent of acetonitrile–10 mM ammonium acetate buffer (50 : 50 v/v, pH 6.0, 0.8 ml min^{-1}).

Inhibition experiments

The polymer particles (3.0 mg) were incubated with atrazine (160 and 320 μM) and ametrin (0–80 μM) for 50, 100, 150 and 200 min at 40 °C. After incubation, the suspensions were filtered and the filtrates (1.0 ml) were dried *in vacuo.* The residues were dissolved in acetonitrile (1.0 ml) and analyzed by HPLC in the same conditions as for the kinetic experiments.

Catalytic activities for various triazine substrates

The polymer particles (3.0 mg) were incubated with atrazine, simazine, propazine and cyanazine (250 μM at 40 °C) in methanol–chloroform (1.5 ml, 1 : 9, v/v) for 12 h. After incubation, the suspensions were filtered and the filtrates (1.0 ml) were dried *in vacuo.* The residues were dissolved in acetonitrile (1.0 ml) and analyzed by HPLC in the same conditions as for the kinetic experiments.

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9 Elemental analysis data of IP(T2)(wt%): C, 84.80; H, 7.7.93; N, 0.26; S, 0.82.