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Target specific sample preparation from aqueous extracts with molecular imprinted polymers

Stephen E. Moring *, Osborne S. Wong, John F. Stobaugh

Higuchi Biosciences Center, Center for Bioanalytical Research, 2099 Constant Avenue, Lawrence, KS 66047-3729, USA

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Abstract

In this paper we report a method for the synthesis of molecular imprinted polymers for use in sample preparation with aqueous biological materials. Highly cross-linked bulk polymers were synthesized in the presence of the template molecule, 2,6-pyridinedicarboxylic acid (DPA) using acrylamide (ACD) and 4-vinylpyridine (VP) as functional monomers. Conditions are described for the optimization of the template complex with temperature, copolymer mixture and crosslinker type. Selective binding of the template molecule is demonstrated in comparison to structural isomers and analogs for molecular imprinted polymers (MIPs) synthesized with three different crosslinkers, ethyleneglycol dimethacrylate (EGDMA), bisacrylamide and *N*,*N*′-1,3-phenylene bismethacrylamide (PBMA). The chromatographic capacity factors and selectivities for a series of structural analogs were compared. Molecular imprinted polymers prepared with equimolar ratios of ACD and VP and either PBMA or bisacrylamide resulted in highly selective binding for the template versus analogs with similar structure and chemistry. Multiple molecular dissociation constants were measured with the maximum binding capacities for EGDMA, PBMA and bisacrylamide measuring 17, 27 and 90 μmol/g, respectively.

Keywords: Molecular imprinted polymer; Sample preparation; Dipicolinic acid; 2,6-Dicarboxypyridine

1. Introduction

Isolation and concentration of analytes of interest from complex sample matrices or highly diluted samples, such as water or air, presents a challenge for sample preparation methods development. Solid phase extraction techniques utilizing reverse phase sorbents have been successfully employed for sample clean up and enrichment. Since RP-SPE columns are nonspecific, interfering substances originating from the sample matrix can be problematic. Molecular imprinted polymers (MIP) can be used for the highly selective isolation of specific analytes for sample preparation when used as MIP solid phase extraction media [1–4]. The potential value of this technology lies in the ability of selectively isolating specific biochemical compounds or their structural analogs from a complex sample matrix.

Molecular imprinted polymers are generally prepared by polymerization of functional

* Corresponding author. Tel.: +1-785-864-3834; fax: +1-785-864-5097.
E-mail address: smoring@ukans.edu (S.E. Moring).

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monomers and a crosslinker in the presence of a template molecule in a porogen solvent system. In one approach the template and monomer/crosslinker form a self-assembled complex in solution prior to polymerization [5]. An alternative to self-assembly is the synthesis of a weakly covalently linked template–vinyl monomer. The monomer(s) and a crosslinker form a highly crosslinked polymer with a pore structure that is determined by the mass/volume ratio and solvating characteristics of the porogen. Fig. 1 illustrates an idealized scheme for non-covalent molecular assembly and synthesis of the type of polymer described in this report. The resulting polymer is ground, sieved and extracted to disassociate the bound template. In the case of a covalently linked template, mild hydrolysis conditions are sufficient for the release of the template molecule. In either case the produced MIP can be used for various purposes such as sample preparation, chromatographic media, biosensors, exclusion membranes, etc. [6–8].

The majority of studies have demonstrated molecular selectivity with imprinted polymers formed in non-polar solvents (porogens). Mosbach et al. has shown various examples of MIPs prepared from functional monomers (e.g. methacrylic acid (MAA) and acrylamide (ACD)) and a cross-linking copolymer (e.g. ethyleneglycol dimethacrylate (EGDMA)) in porogens such as chloroform, toluene, tetrahydrofuran and acetonitrile [9–11]. With MIPs prepared with this approach only fairly apolar templates can be used in non-aqueous sample extracts. Hence, MIPs prepared for use in sample preparation of biological and environmental samples preclude the processing of samples in aqueous media. The requirement of the use of organic extraction steps is highly disadvantageous. Recent research has demonstrated the synthesis of MIPs in polar porogens and their use for isolation of template analogs from aqueous extracts [12,13].

The objective of this research is to demonstrate feasibility for synthesis of MIPs to a polar template molecule for use in sample preparation with aqueous samples. We have attempted to select conditions for the optimal activity with the use of different functional and cross-linking copolymers and polymerization temperature. The template molecule chosen for this study is 2,6 dicarboxypyridine (dipicolinic) acid, a metabolic marker for Bacillus spores [14]. This analyte is zwitterionic with pK_a at 2.32 and 4.53, and is soluble only in polar solvents. For preparation of an active MIP we have investigated the synthesis of polymers in a porogen solution of aqueous methanol with a mixture of acrylamide (ACD) and 4-vinylpyridine (VP) and various crosslinkers, EGDMA, *N*,*N*′-1,3-phenylene bismethacrylamide (PBMA) and *N*,*N*′-methylene bisacrylamide (BIS).

2. Experimental

2.1. Instrumentation

Proton NMR spectra were obtained on Bruker Model AM-500 instrument (Bruker Instruments, Billerica, MA, USA). Absorbance measurements and spectra were recorded on a Hewlett–Packard Model 8451A Diode Array Spectrophotometer (Agilent Technology, Palo Alto, CA, USA). High powered liquid chromatography (HPLC) was carried out on a Shimadzu LC-6A Solvent Delivery System (Shimadzu Scientific Instruments, Columbia, MD, USA), ABI 491 Dynamic Mixer/Injector and ABI 757 Absorbance Detector (Applied Biosystems, Foster City, CA, USA). Calorimetric analyses (melting point determination) were made with a Perkin Elmer Pyris 1 Differential Scanning Calorimeter (Perkin–Elmer, Norwalk, NJ, USA).

2.2. Materials

Methanol, tetrahydrofuran, dimethyl formamide and hydrochloric acid (HCl) were purchased from Fisher Scientific Company (St. Louis, MO, USA). Water (18 MΩ) was purified with a Barnstead NANO® System (Barnstead/Thermolyne, Dubuque, IA, USA). MAA, methacrylamide (MACD), EGDMA, *N*,*N*′-azobisisobutylronitrile (AIBN), 2,6-pyridinedicarboxylic acid (DPA), methacryloyl chloride, and 1,3-diaminobenzene were obtained from Aldrich Chemical Co. (Milwaukee, WI, USA). ACD and BIS

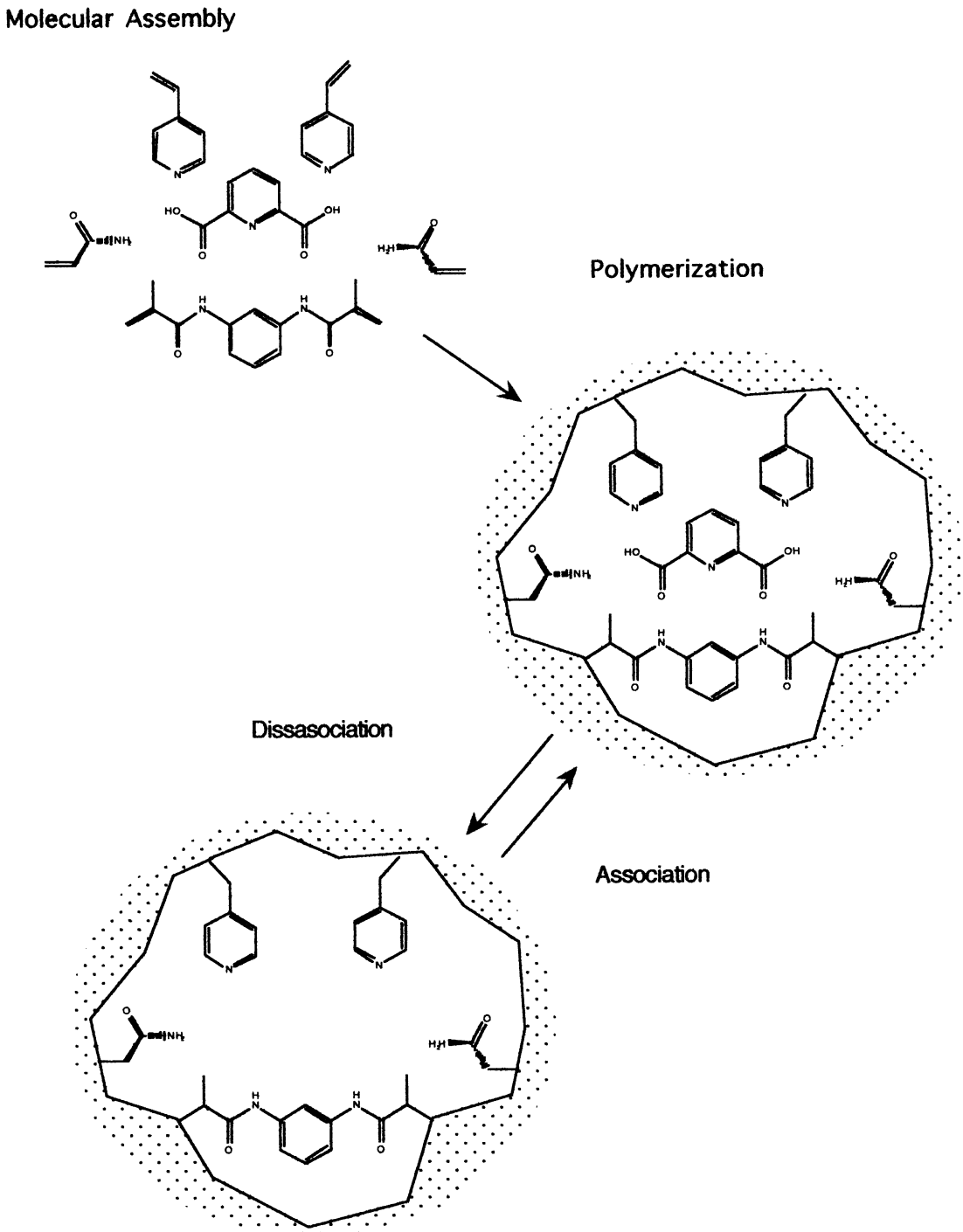

Fig. 1. Idealized molecular assembly, polymerization of a DPA molecular imprinted polymer and disassociation of template.

USA). ACD and BIS were purchased from Bio-Rad Laboratories (Hercules, CA, USA).

2.3. Synthesis of *N,N-1,3-phenylenebismethacrylamide (PBMA)*

PBMA was synthesized according to a procedure by Shea et al. [15]. To a solution of methacryloyl chloride (10 ml, 102 mmol) in 200 ml of acetonitrile at 4 °C, a solution of 1,3-diaminobenzene (12.0 g, 111 mmol) was added dropwise under an atmosphere of argon gas. The mixture was stirred at 22 °C for 3 h. The salts were filtered in a Buchner funnel with Whatman #1 filter paper (Whatman, Maidstone, UK) and washed with 20–30 ml of acetonitrile warmed to a temperature of approximately 40 °C. The filtrate was evaporated to 100 ml under vacuum with roto-evaporator apparatus and refrigerated. The PBMA crystals were filtered and washed in cold (4 °C) acetonitrile. The PBA was recrystalized in acetonitrile; 6.7 g yield, mp 149.2 °C; 1H NMR (300 MHz, CD_3OD): δ, 7.912 (1H, $J = 2.0$, Ar*H*), 7.35 (2H, Ar*H*), 7.28 (1H, Ar*H*), 5.78 (2H, vinyl), 5.50 (2H, vinyl), 2.02 (6H, methyl).

2.4. Synthesis of polymers

Synthesis of experimental polymers was carried out with a 15–24 mmol crosslinker (EGDMA, 20.0 mmol; BIS, 24.3 mmol; or PBMA, 15.2 mmol), 1–4 mmol of functional monomers (MAA, VP, MACD, ACD), 1 mmol DPA and 0.25 mmol AIBN in 8–12 ml porogen solution. Porogen solutions were purged with helium prior to use. Monomer, crosslinker, initiator and porogens were mixed in a glove box under argon. Polymerizations were carried out in an oil bath regulated from 40–60 °C for 24–48 h. The finished polymers were coarsely ground with a mortar and pestle. The polymers were extracted in 100 ml of methanol in a Soxhlet apparatus for 4 h. The polymers were further extracted to remove DPA by successive washes in five volumes of a mixture of methanol and 100 mM HCl (9:1 v/v) until the UV absorbance at 270 nm measured below 0.01 AU.

2.5. Binding studies

The binding capacity of DPA for experimental polymers in comparison to control polymers (those prepared in the absence of template) was measured by suspension in 1.0 ml of 10 μg/ml DPA solutions with 50 mg of polymer. The polymers were air dried in a fume hood for 18 h prior to use and resuspended in various test buffer solutions and washed successively to reduce background absorbance. The assays were carried out in 1.5-ml polypropylene microcentrifuge tubes with incubation of DPA solution for 1 h with frequent mixing. The binding of DPA was determined by measurement of the absorbance difference of an equivalent dilution of DPA standard solution and the supernatants after centrifugation at 7000 rpm for 3 min. Absorbance measurements were made by calculation of the absorbance difference, ΔAU of 270–300 nm.

2.6. Chromatographic evaluation

The measurement of chromatographic capacity factors were used to determine the relative binding of DPA analogs to experimental polymers (Fig. 2). Polymer particles were ground and sieved to 25–50 μm fractions. Slurries of polymer were prepared in 50% aqueous methanol and packed into 20 × 3 mm i.d. stainless steel guard columns (Higgins Analytical). Aliquots (20 μl) of various DPA analog solutions (50 μg/ml) were injected and eluted with an isocratic mobile phase of 40% methanol in a 0.1% aqueous TFA at a flow rate of 1.0 ml/min with detection at 270 nm. The capacity factors (k') were calculated by the equation, $k' = (t_R - t_0)/t_0$, where t_R is the retention time for the test analyte and t_0 is the void time of acetone. Binding selectivity was calculated as the ratio (α) of the k' value for the template with respect to the test analogs (k'_{temp}/k'_{anal}).

2.7. Determination of binding constants by Scatchard analysis

Binding constants were calculated by extrapola-

tion measurements of DPA binding from 1 to 900 μM concentrations with 20 mg of each of the tested MIPs. Aliquots (1 ml) of DPA solution were incubated with MIPs for 1 h at 25 ° C. The unbound DPA from supernatants was measured at ΔUV absorbance at 270–300 nm. The binding constants were determined from the equation, $B/[\text{Free}] = (B_{max} - B)/K_D$, where K_D is the equilibrium dissociation constant, and B_{max} is the maximum number of binding sites [16].

3. Results and discussion

3.1. Comparison of polymers synthesized in apolar and aqueous porogens

The first phase of experimentation for the synthesis of imprinted polymers to DPA was the preparation of polymers using various molar percentages of ACD as a functional monomer and EGDMA as a cross-linking copolymer. Table 1

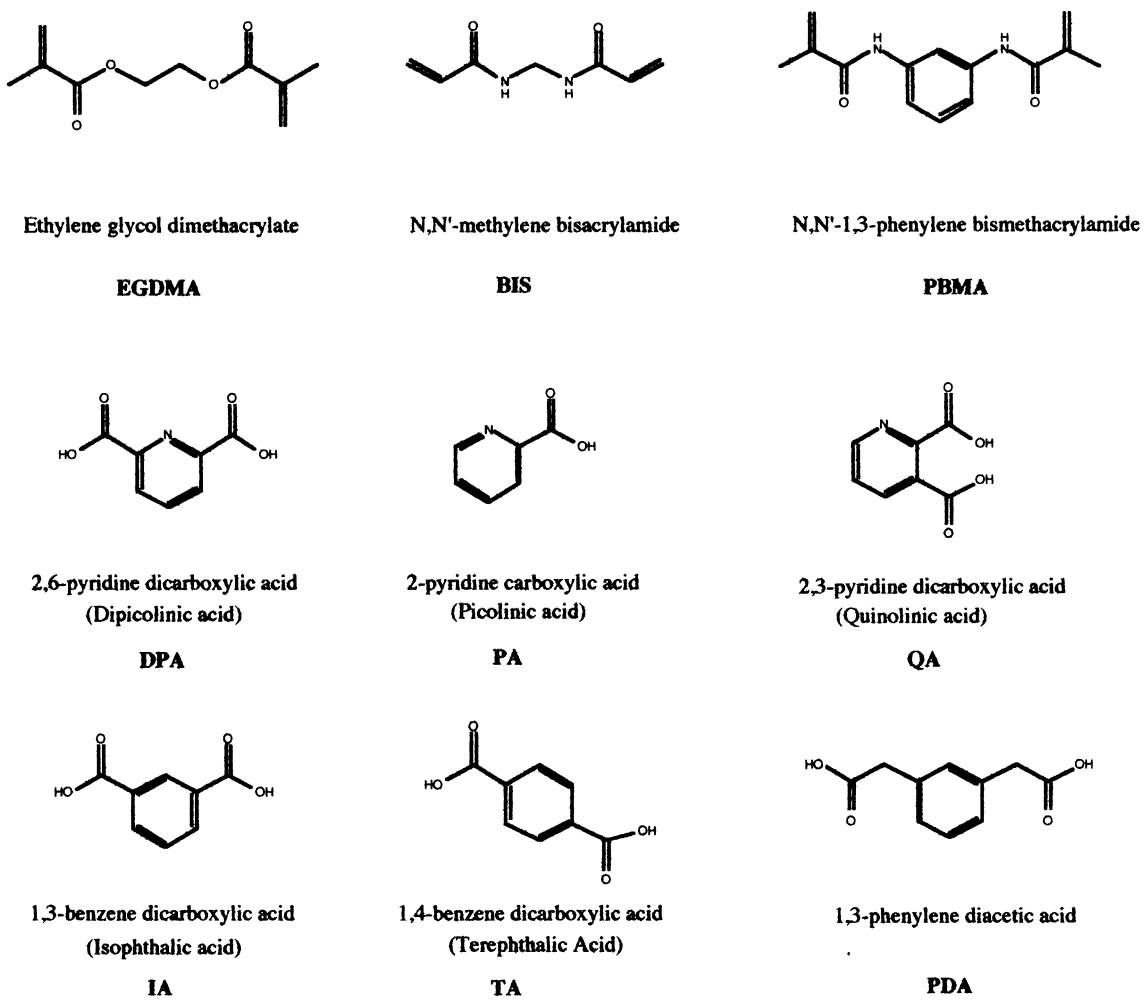

Fig. 2. Cross-linking copolymers, and structural analogs used for assessing chromatographic selectivity of experimental MIPs.

Table 1
MIP experimental design for the synthesis of MIPs

	Template (mol%)	Functional monomer (mol%)			Cross-linker (mol%)	Porogen	Temperature (°C)
	DPA/control	MAA	ACD	4-VP	EGDMA		
1	4/+	–	8	–	79–91	MeOH	50–55
2	4/+	–	8	–	”	DMF	”
3	4/+	–	12	–	”	THF	”
4	4/−	–	16	–	”	THF	”
5	4/−	12	–	–	83–87	THF/DMF*	”
6	4/−	4	4	4	”	” ”	”
7	4/+	–	8	4	”	” ”	”
8	4/−	–	12	4	79–83	MEOH/H_2O**	25–45/55–60
9	4/+	–	8	8	”	” ”	”
10	4/−	–	8ψ	8	”	” ”	”
11	4/−	–	4	12	”	” ”	”
12	4/+	–	0	16	”	” ”	”

*, THF/DMF 97:3 v/v; porogen:monomer, 3:1 v/w; **, MeOH/H_2O 4:1 v/v; porogen:monomer, 2:1 v/w; ψ, MACD.

shows the experimental design parameters used for these and subsequent experiments with MAA and VP as functional monomers. Comparison of DPA binding to imprinted polymers prepared in non-aqueous porogens to those of control polymers, prepared in the absence of DPA, resulted in little or no measurable binding to processed MIPs.

In the second phase of experiments, methanol/water (4:1 v/v) was used as a porogen. The functional monomers (ACD, MACD, and VP) were used exclusively with EGDMA. Table 2 shows the results of DPA binding with MIPs prepared with varying ratios of ACD to VP from two different syntheses. The greatest activity was observed with a concentration of VP exceeding 8 mol%. Substituting MACD for ACD resulted in slightly lower activity. An interesting difference in binding affinity was observed for polymers synthesized under poorly regulated temperature with an non-insulated oil bath. Under these conditions the reaction vessels contained a temperature gradient with the lower end measuring approximately 10 °C below the set temperature. The highest DPA binding resulted from MIPs synthesized at an initial polymerization temperature of 40–45 °C for a minimum of 18 h. For subsequent experiments the polymerization temperature was carefully regulated and applied in steps consisting of an initial incubation at 25 °C for 30 min, followed by 40–45 °C for 24 h and 55–60 °C for 24 h. Table 3 shows the relationship of incubation temperature and time with the binding activity of MIPs prepared with the same monomer composition. Clearly the highest activity results from lower temperatures that facilitate a higher order of molecular association with increased hydrogen bonding.

3.2. Comparison of template binding for MIPs with various crosslinkers

The activities of MIPs prepared with different crosslinkers were investigated. The polymers were prepared at a higher monomer to porogen ratio (1:3 w/v) to facilitate dissolution of BIS and PBMA in 80% methanol in water. The freshly mixed monomers and porogen were heated briefly to 50–60 °C to bring BIS and PBMA into solution. Reaction mixtures were incubated at 40–45 °C for 26 h followed by 50–55 °C for 26 h. Table 4 shows the percent binding of DPA in various aqueous buffer/methanol solutions. The binding of DPA was greatest for polymers synthesized with PBMA. The binding of DPA for the various control polymers averaged at ~20%. This value is relatively high and may be attributed to primarily a nominal 15–20% dilution of the

Table 2
Binding of DPA to VP–ACD MIPs

Experiment	Polymer composition (mol%)					Percent DPA bound			
	DPA	ACD	VP	EGDMA	Temperature	Aqueous buffer*	60% Aqueous MeOH*	90% Aqueous MeOH*	10% Aqueous HCl/MeOH**
8	4	12	4	79	NR	47.1	27.7	31.0	17.9
9	4	8	8	79	NR	63.3	34.9	28.4	14.4
9 c	0	8	8	83	NR	23.7	12.2	35.2	22.9
9′	4	8	8	79	R	29.7	18.0	22.2	13.6
9′ c	0	8	8	83	R	12.7	11.6	16.3	14.1
11′	4	4	12	79	R	29.7	18.8	23.6	15.3
12	4	0	16	79	NR	60.9	39.0	38.3	15.7
12 c	0	0	16	83	NR	18.1	12.1	26.4	17.3

Polymerization, 1h (25 °C), 2 h (45 °C); 20 h (55–60 °C); NR, oil bath not regulated (non-insulated); R, temperature regulated; porogen: methanol/water, 4:1 v/v; monomer to porogen ration 2:1 w/v. *, Aqueous buffer, ~40 mM Na–phosphate, pH 7.0, 0.1% tween 20; **, 100 mM HCl.

Table 3
Activity of DPA MIPs vs. polymerization temperature and time

Temperature/time	Percent DPA bound in aqueous buffer*	
	DPA MIP	Control
2 h @ 45–50 °C	29.7	12.7
24 h @ 45 °C	46.2	15.5
18 h @ 40 °C	68.6	17.1

MIP composition: EGDMA:ACD:VP:DPA:AIBN 20:2:2:1:(0.3). *, See Table 2.

added DPA standard solution by solvent in the wetted polymer pellets. Prior to testing the polymers were washed in test buffer solution. The volume of buffer solution absorbed by the polymers were between 15 and 20% of the assay buffer volume. Despite the contribution of dilution, some non-specific binding of DPA is likely to be occurring in controls tested with aqueous buffer.

3.3. Selectivity of imprinted polymers synthesized from various cross-linker copolymers

A comparison of the chromatographic retention capacity and selectivity for various phenyl carboxylic acids and DPA is listed in Table 5. MIPs prepared with EGDMA exhibited the lowest chromatographic retention compared with the others. The selectivity was low and unusual, since higher retention was observed for the non-pyridine benzene mono- and dicarboxylic acids. The EGDMA polymer possesses weak anion exchange properties with a generalized selectivity that appears to be generated as a result of imprinting with DPA. It is interesting that both PA and PDA exhibited lower retention than the template. Both MIPs prepared from BIS and PBMA resulted in very high retention for the template and selectivity over the various analogs tested. The overall highest selectivity was measured for the BIS MIP that demonstrated lower retention for the phthalic acid analogs (TA & IA). Compared with the PDMA MIP, the BIS MIP exhibited a lower specificity for the template versus its control (3.8 vs. 2.2, respectively).

3.4. Scatchard analysis of template binding to imprinted polymers

The binding capacities and dissociation constants were calculated for the MIP prepared from different crosslinkers by extrapolation of data measurement of DPA concentration ranging from 1 to 900 μmol. Scatchard plots of the ratio of bound DPA/freely soluble versus bound DPA revealed the presence of multiple binding sites. Table 6 shows the binding constants for MIPs prepared from various crosslinker copolymers. The presence of multiple binding sites is indicative of the instability of the template–functional monomer complex that is the product of non-covalent self-assembly during polymerization.

Table 4
Binding of dipicolinic acid to VP–ACD polymers

Polymer	Percent DPA bound			
	Aqueous buffer*	60% Aqueous MeOH	90% Aqueous MeOH	10% Aqueous HCl**
PBMA, DPA MIP	89.2	73.6	–	24.7
PBMA control	24.1	20.1	34.5	22.8
BIS, DPA MIP	83.0	69.3	41.8	23.4
BIS control	28.2	16.7	29.1	20.4
EGDMA, DPA MIP	68.6	35.3	34.2	12.5
EGDMA control	17.1	15.0	22.0	15

Monomer composition: crosslinker, 79–83 mol%; VP, 8 mol%, ACD, 8 mol%, (DPA, 4 mol%) and AIBN, 1 mol%. Porogen: methanol/water, 4:1 v/v; monomer:porogen, 3:1 w/v. *, Aqueous buffer: 40 mM sodium phosphate, pH 7.0, 0.1% tween 20. **, 100 mM HCl.

Table 5
Selectivity of DPA MIPs with different crosslinkers measured by chromatographic capacity factors

	EGDMA			BIS			PBMA		
	$k'_{Control}$	k'_{MIP}	α	$k'_{Control}$	k'_{MIP}	α	$k'_{Control}$	k'_{MIP}	α
DPA	0.273	2.28 ($\pm$0.056)[a]	–	5.64 ($\pm$0.184)[a]	12.38 ($\pm$1.05)[a]	-	3.31 ($\pm$0.998)[a]	12.61 ($\pm$0.728)[a]	–
PA	0.021	0.050	45.6	0.183	0.207	60.0	0.276	0.503	25.0
QA	0.096	0.771	3.0	2.68	3.64	3.4	1.09	3.69	3.4
TA	0.656	6.24	0.36	1.75	1.85	6.7	2.51	2.98	4.2
IA	0.584	5.04	0.45	1.36	1.49	8.3	2.84	3.10	4.1
PDA	0.270	0.970	2.4	0.592	0.250	49.5	1.49	1.51	8.4
BA	0.503	4.45	0.51	–	–	–	1.00	1.17	10.8

[a] S.D., $n = 3$.

Table 6
Binding Constants for DPA MIPs

	EGDMA MIP	PBMA MIP	BIS MIP
B_{max}	17.0 μmol/g	27.0 μmol/g	90.0 μmol/g
K_{D1}	4.05×10^{-7} M	1.58×10^{-6} M	2.36×10^{-6} M
K_{D2}	5.2×10^{-8} M	7.8×10^{-8} M	1.2×10^{-7} M
K_{D3}	–	–	1.85×10^{-8} M

4. Conclusion

The DPA imprinted polymers with BIS and PDMA crosslinkers demonstrated very high selectivity in regard to their potential use as a sample preparation media. The selectivity appears to be dependent on a combination of ion pairing and hydrogen bonding of the template to both the functional monomer and crosslinker. The highest binding specificity to the template was measured with the PDMA-MIP. It is interesting to note the molecular orientation afforded by the use of PDMA (Fig. 1) with respect to the other crosslinkers. The binding activity is also highly dependent on the initial polymerization temperature, which is again indicative of the importance of highly specific hydrogen bonding in the template monomer molecular assembly.

A solid phase extraction media of BIS of PDMA MIPs developed for an application involving the selective trapping and concentration of DPA from aqueous extracts of aerial spore samples would significantly enhance the efficiency and sensitivity of the type of microbial assays described by Snyder et al. [14]. These polymers efficiently bind this template in aqueous solution and can be easily eluted in acidified methanol. The total binding capacities and the degree of multiplicity of binding sites of these MIPs require further study. Further steps for the optimization of the MIP synthesis include the determination of the effect of porogen pH, the presence of complexing metal ions, polymer pore size with the manipulation of monomer to porogen ratio, and the use of other water miscible organic solvents.

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