Target Analogue Imprinted Polymers with Affinity for Folic Acid and Related Compounds

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Abstract: Two approaches to synthesize molecularly imprinted polymers with affinity for folic acid and other substituted pteridines have been compared. In the first approach, the folic acid analogue methotrexate was used as template and functional monomers capable of generating selective binding sites were searched in a miniaturized screening system based on binding assessment in the batch mode. Highest selectivity was seen using 2-vinylpyridine as functional monomer, which was confirmed in the chromatographic mode for a batch synthesized on a gram scale. However, the retentivity and selectivity of this phase were insufficient for anticipated applications. In a second approach, using methacrylic acid as the functional monomer, organic soluble inhibitors for the enzyme dihydrofolate reductase were used to develop sites complementary toward the pteridine substructure. This resulted in materials showing enhanced selectivity for substituted pteridines when evaluated by HPLC. Thus, methotrexate and leucovorine were selectively retained in mobile phases of either low or high aqueous content, thus showing the typical bimodal retention behavior of previously reported MIPs. In organic mobile-phase systems, the inhibitor used as template had an influence on the retentivity and selectivity of the MIP. The polymer imprinted with trimethoprim retained all folic acid analogues strongly and showed the highest selectivity among the MIPs in an organic mobile-phase system. This was supported by Scatchard analysis resulting in biphasic plots and a quantitative yield of high-energy binding sites. All templates were shown to associate strongly with MAA in $CDCl_3$, the strength of association correlating roughly with the template basicity and the selectivity observed in chromatography. Nonparallel complexation-induced shifts indicated formation of 1:2 template monomer complexes at concentrations corresponding to those of the prepolymerization solutions.

Introduction

The synthesis of artificial receptors capable of recognizing complex biomolecules in water remains an important challenge in chemistry.[1] The approaches so far taken have involved low molecular weight hosts designed on the basis of molecular modeling,[2−4] the generation of peptide or nucleic acid libraries, followed by screening of these to identify target binding sequences,[5] or self-assembled recognition elements obtained by allowing hosts to assemble in the presence of the target or substructures of the target molecule.[6]

This latter approach has been used to synthesize molecularly imprinted polymers (MIPs) for a large number of low molecular weight target molecules, and these polymeric receptors have been shown to recognize their targets with affinities and selectivities in same order as antibodies generated for the same target structure.[7−12] Despite this impressive trait, MIPs are inferior to antibodies in some important respects. One of these is the poor aqueous compatibility, reflected in nonspecific adsorption and poor recognition of targets in water. There is also a lack of general approaches to generate sites for more complex biomolecules. In this case, the polymer may need to be synthesized in aqueous media to solublize the target and to stabilize it in a conformation close to its native low-energy conformation. However, in water, the strong hydration forces and the polar environment prevent stable electrostatic interactions between the target and the functional monomers that are commonly used in molecular imprinting.

Despite these problems, advances have been made. Introduction of functional monomers targeted toward specific functional groups via hydrogen-bonded ion pairs,[13,14] donor−acceptor interactions,[15] hydrophobic forces,[16−18] metal ion-mediated

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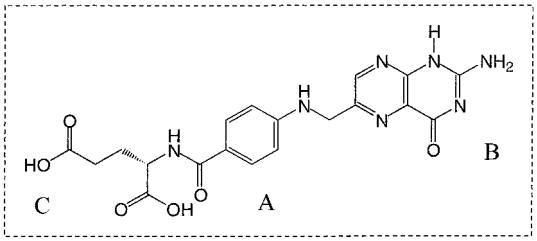


Figure 1. Substructure approach for recognition of folic acid and analogues by molecular imprinting. (A) Folic acid or analogue as template; (B) pteridines as templates; (C) N-protected glutamic acid as template.

binding,[19−21] or a combination of noncovalent and covalent attachments[22] may effectively allow subsequent recognition of polar biomolecules to take place in aqueous media. In addition, general purpose monomers may be used to generate sites for proteins showing impressive selectivities.[23]

An alternative approach is to use organic soluble analogues complementary to substructures of the target.[24] Although the resulting sites are by consequence less specific for the target, this strategy uses simple templates that are soluble in aprotic solvents. This may result in sites complementary to the substructure that still bind the larger biomolecules with significant affinities. The corresponding binding energy may be large enough to overcome rotational barriers for fitting the larger substrate to the sites.

In this work, we have compared such approaches with traditional approaches to generate sites for folic acid and analogues (Figure 1). Folic acid is a water-soluble B vitamin derived from its conjugates, folate polyglutamates, and is found in foods such as green leafy vegetables, organ meats, and some fresh fruits. It is converted in the liver and plasma to tetrahydrofolate.[25]

The design of the sites was inspired by the active site structure of the enzyme dihydrofolate reductase (DHFR). This reduces dihydrofolate to tetrahydrofolate which, by donation of single carbon fragments, plays a critical role in the biosynthesis of amino acids, DNA, and RNA. The inhibitors of this enzyme (trimethoprim (TMP) and trimetrexate (TRX) in Chart 1 and methotrexate (MTX) in Chart 2) have found important use in medicine. For instance, MTX is used in cancer therapy, since it preferentially slows down the cell growth of rapidly growing cancerous cells. In the site design, it is interesting to study the crystal structure of this inhibitor docked in the active site of the enzyme.[26] This may suggest how to initially design the imprinted site. Thus, it is seen that MTX binds to the site via

Chart 1

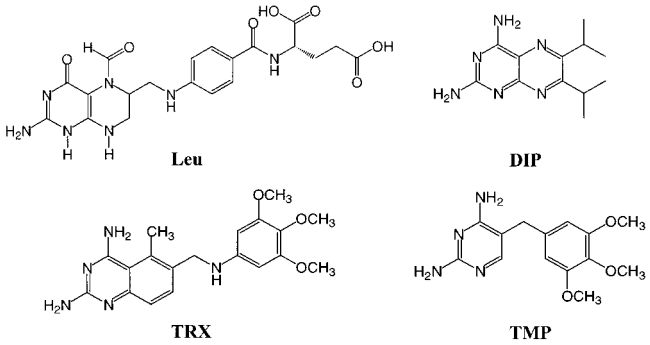
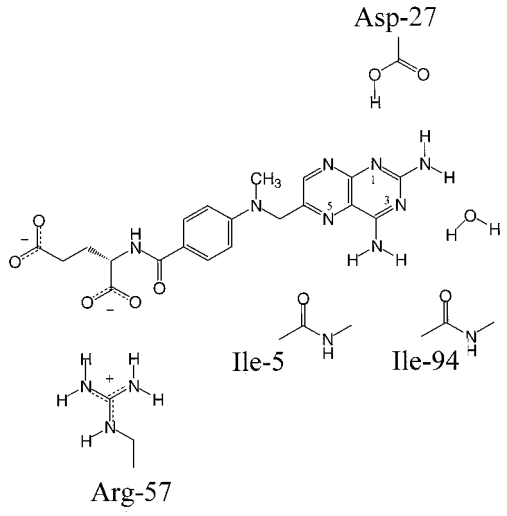


Chart 2



hydrogen bonds between Asp27 and the N1 of the pteridine, in combination with a salt bridge between the glutamic acid substructure and an arginine guanidine functional group (Chart 2).[27] This suggests that a site for the pteridine substructure can be formed by using methacrylic acid (MAA) as functional monomer and the corresponding inhibitor as template. Likewise, the glutamic acid substructure can be targeted using other functional monomers and N-protected glutamic acid as template. Using this substructure approach, materials have been prepared resulting in enhanced retentions of folic acid and analogues in water when the polymers were evaluated in the chromatographic mode.

Experimental Section

Chemicals. Methotrexate (MTX, (+)-amethopterine), leucovorine (Leu, *dl*-L-form of folinic acid as its calcium salt), folic acid (Fol), 2,4-diamino-6,7-diisopropylpteridine (DIP), methacrylamide (MAAM), methyl methacrylate (MMA), methacrylic acid (MAA), 2-hydroxyethyl methacrylate (HEMA), itaconic acid (ITA), *N*-vinyl-2-pyrrolidone (NVP), trifluoromethacrylic acid (TFM), ethylene glycol dimethacrylate (EDMA), dichloromethane, chloroform, and *N*-methylpyrrolidone were purchased from Sigma Aldrich Chemie Gmbh (Deisenhofen, Germany). Trimetrexate glucuronate (Neutrexin) was a gift from U.S. Bioscience Inc. and was transformed to the free base form by extraction with Na_2CO_3 as follows. Trimethrexate glucuronate (250 mg) was dissolved in water (100 mL). After the pH was adjusted to 11 with Na_2CO_3, trimetrexate was extracted into dichloromethane (3 × 300 mL). Trimethoprim (TMP) was obtained as a gift from Hoffman la Roche

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Ltd. (Basel, Switzerland). 4-Vinylpyridine (4-VPy), 2-vinylpyridine (2-VPy), acetonitrile, and the buffer salts, NaH_2PO_4 and Na_2HPO_4, were purchased from Merck (Darmstadt, Germany). Acetic acid was obtained from Fluka BioChemika (Neu-Ulm, Germany).

The azoinitiator 2,2′-azobisisobutyronitrile (AIBN) was obtained from Janssen. Prior to use, AIBN was purified by recrystallization from methanol. EDMA was purified by extraction with 10% NaOH, washing with brine, drying over anhydrous magnesium sulfate, and subsequent distillation under reduced pressure. 2-VPy, 4-VPy, and MAA were purified by distillation under reduced pressure; other monomers were used as received, but stored under cool and dry conditions on appropriate molecular sieves. The porogens were all distilled under a positive nitrogen atmosphere. The deuterated chloroform used in the NMR titrations was dried by treatment with activated alumina and stored on molecular sieves. Other solvents were of HPLC grade and stored on molecular sieves. The buffer solutions (0.2 mM) were prepared by dissolving the salts in bidistilled water and adjusting the pH with hydrochloric acid (0.1 M).

Apparatus. The glass vials (1.5 mL) with rubber septa used as polymerization reactors were purchased from Supelco. All chromatographic evaluations were performed using a Hewlett-Packard instrument (HP1050 or equivalent) equipped with a quaternary pump, an autosampler, a diode array detector, and an HP work station. Unless otherwise mentioned, the flow rate was 1 mL/min and the separations run at room temperature. The NMR spectra were recorded on a Bruker 400 MHz NMR spectrometer.

Small-Scale MIPs (MiniMIPs).[28] *Synthesis*. Two mother solutions (with or without template) were prepared by mixing EDMA (1.7 mL), AIBN (25 mg), MeCN (2.5 mL), and NMP (1.25 mL). For the MIP series, MTX (10 mg) was added as template. A total of 100 μL of each mother solution was then dispensed into 1.5-mL glass vials followed by addition of one of the functional monomers (MAA, 4-VPy, MAAM, TFM, NVP, ITA, 2-VPy, MMA, HEMA; 40 mol % total monomer). To the vials containing MAAM, NVP, 2-VPy, MMA, and HEMA, 10 μL of NMP was also added. Each vial was then sealed with a rubber septum and purged with nitrogen for 5 min. The vials were then heated for 24 h at 60 °C in an oven.

Template Extraction and Rebinding Experiments. After polymerization, the release of the template in the porogenic mixture was quantified. For this purpose, 1 mL of MeCN/NMP (2:1, v/v) was dispensed to each vial, the vials were shaken, and after 4 h, the free MTX in the supernatant was quantified by reversed-phase HPLC using a 10 μL injection volume, a C18 reversed-phase column (Prodigy 5 μm, ODS3, 125 × 4.6 mm), with the mobile phase MeCN/potassium phosphate buffer 0.02 M (pH 7.4) (10:90, v/v), UV detection at 288 nm, and MTX as an external standard. To the vials were then added 1 mL of HOAc, and the quantity of MTX released was again assessed after 4 h as above. The rebinding experiment was then performed by adding 1 mL of a solution of MTX (0.16 mM) in MeCN/NMP (2:1, v/v) to each vial, and the concentration of free MTX was determined after 1, 26, and 76 h.

Normal-Scale MIP Synthesis. MIP and blank polymers were prepared with the compositions given in Table 1. In the case of polymer PDIP, MAA (0.30 mL, 3.4 mmol), EDMA (3.6 mL, 18 mmol), AIBN (40 mg), and DIP (50 mg, 0.20 mmol) were mixed in CH_2Cl_2 (7 mL) and then transferred to glass polymerization tubes (14 mm i.d.). Each solution was then degassed with nitrogen for 15 min, and the tubes sealed and heated at 60 °C for 8 h. After the polymerization, the monolith was crushed and sieved in order to obtain particles with a diameter between 25 and 36 μm. These were then washed consecutively with 3 × 40 mL of MeOH/H_2O (1:1), MeOH, MeOH/HOAc (9:1), and MeOH and thereafter dried.

Chromatographic Evaluations. The particles (25−36 μm) were slurried in methanol/water (80:20) and packed into HPLC columns (25 mm × 4 mm) at a maximum pressure of 300 bar using a compressed gas-driven slurry packer. The columns were then fitted to an HPLC system and conditioned using MeCN/H_2O/HOAc (92.5:2.5:5, v/v/v) as mobile phase.

Thereafter, the polymers were tested by comparing the capacity factors (k') estimated graphically from the peak maximums obtained by injecting 10 μL of solutions (1 or 10 mM) of the template (dissolved in acetonitrile), MTX (dissolved in H_2O/NaOH 0.01 M, 80:20), Leu, and Fol (dissolved in water) on the blank and imprinted columns. The void markers were acetone (mobile phase: MeCN and MeCN/H_2O/HOAc (92.5:2.5:5, v/v/v)) or ethanol/water (1:1). The UV detection wavelengths were as follows: 288 nm for MTX and Leu, 242 nm for DIP, and 247 nm for TMP and TRX.

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Table 1. Composition of Folic Acid Analogue Imprinted Polymers[a]

polymer code	template	mol %	functional monomer	mol %	solvent	V_S/V_M[b]
PMTX1	MTX	0.15	2-VPy	40	MeCN/NMP, 2:1	1.6
PMTX1BL			2-VPy	40	MeCN/NMP, 2:1	1.6
PMTX2	MTX	0.15	MAA	40	MeCN/NMP, 2:1	1.6
PMTX2BL			MAA	40	MeCN/NMP, 2:1	1.6
PDIP	DIP	0.90	MAA	16	CH_2Cl_2	1.8
PDIPBL			MAA	16	CH_2Cl_2	1.8
PTMP	TMP	0.90	MAA	16	CH_2Cl_2	1.8
PTMPBL			MAA	16	CH_2Cl_2	1.8
PTRX	TRX	0.45	MAA	55	CH_2Cl_2	2.7
PTRXBL			MAA	55	CH_2Cl_2	2.7

[a] The polymers were prepared as described in the Experimental Section using the above monomer compositions and EDMA as cross-linking monomer. After the HPLC assessment, nitrogen elemental analysis on the polymers indicated that at least 90% of the template had been removed. The FTIR (KBr) spectra of the polymers showed the following main bands (cm^{-1}): PDIP 754, 953, 1160, 1261, 1391, 1457, 1478, 1637, 1731, 2958, 2992, 3504; PTRX 756, 959, 1164, 1262, 1390, 1455, 1470, 1636, 1722, 2958, 2992, 3250, 3550; PMTX1 751, 952, 1151, 1249, 1388, 1476, 1570, 1595, 1631, 1731, 2956, 2982, 3469, 3588. No significant difference was seen between the spectra of the imprinted and blank polymers. [b] Ratio of the volume of solvent to the total monomer volume.

Batch Partitioning Experiments. Stock solutions of DIP or TMP (10 mM) dissolved in chloroform or acetonitrile were prepared and diluted in order to obtain solutions with appropriate concentrations: 5, 4, 3, 2.5, 2, 1.5, 1, 0.75, 0.5, 0.25, 0.1, 0.075, 0.05, 0.025, and 0.01 mM. Aliqouts (500 μL) of these solutions were added to glass vials containing 5 mg of MIP or blank polymer. In addition, pure solvent was added to one set of MIP and blank polymer in order to compensate for background drift due to bleeding of template occurring during the period of the experiment. After 20 h, where the vials were shaken at regular time intervals, the amount of free ligand in the supernatant was quantified by HPLC using an external standard for calibration. For the partitioning experiment using acetonitrile as solvent, a C18 reversed-phase column (Prodigy 5 μm ODS3, 125 mm × 4.6 mm) was used. In this case, the mobile phase was acetonitrile/potassium phosphate buffer, 0.2 M, pH 7.4 (50:50, v/v) with UV detection at 242 nm. In the case where chloroform was used as solvent, the quantification was done by flow injection in chloroform. It was not possible to obtain binding isotherms for TRX due to its limited solubility in these solvents.

Template Monomer Titrations Monitored by ^{1}H NMR. Stock solutions of the template (0.01 M) (TRX, 0.005 M) and MAA (0.15 M) (in the titration of TRX, 0.075 M) were prepared by dissolving appropriate amounts of the template and the monomer in $CDCl_3$. Aliquots of the template solution (0.3 mL) were pipetted into NMR tubes, and different volumes of MAA and $CDCl_3$ were added in order to obtain molar ratios of monomer to template ranging from 0 to 15 in a total volume of 0.6 mL. The ^{1}H NMR spectra were recorded and the shifts of the significant protons were taken.

Results and Discussion

The objective of the work was to study and compare materials prepared using templates copying the complete structure or substructures of folic acid, in terms of their selectivity and affinity for related target molecules. Thus, the approaches shown in Figure 1 were compared.

Complementarity toward the Complete Target. The dihydrofolate reductase (DHFR) inhibitor MTX was considered

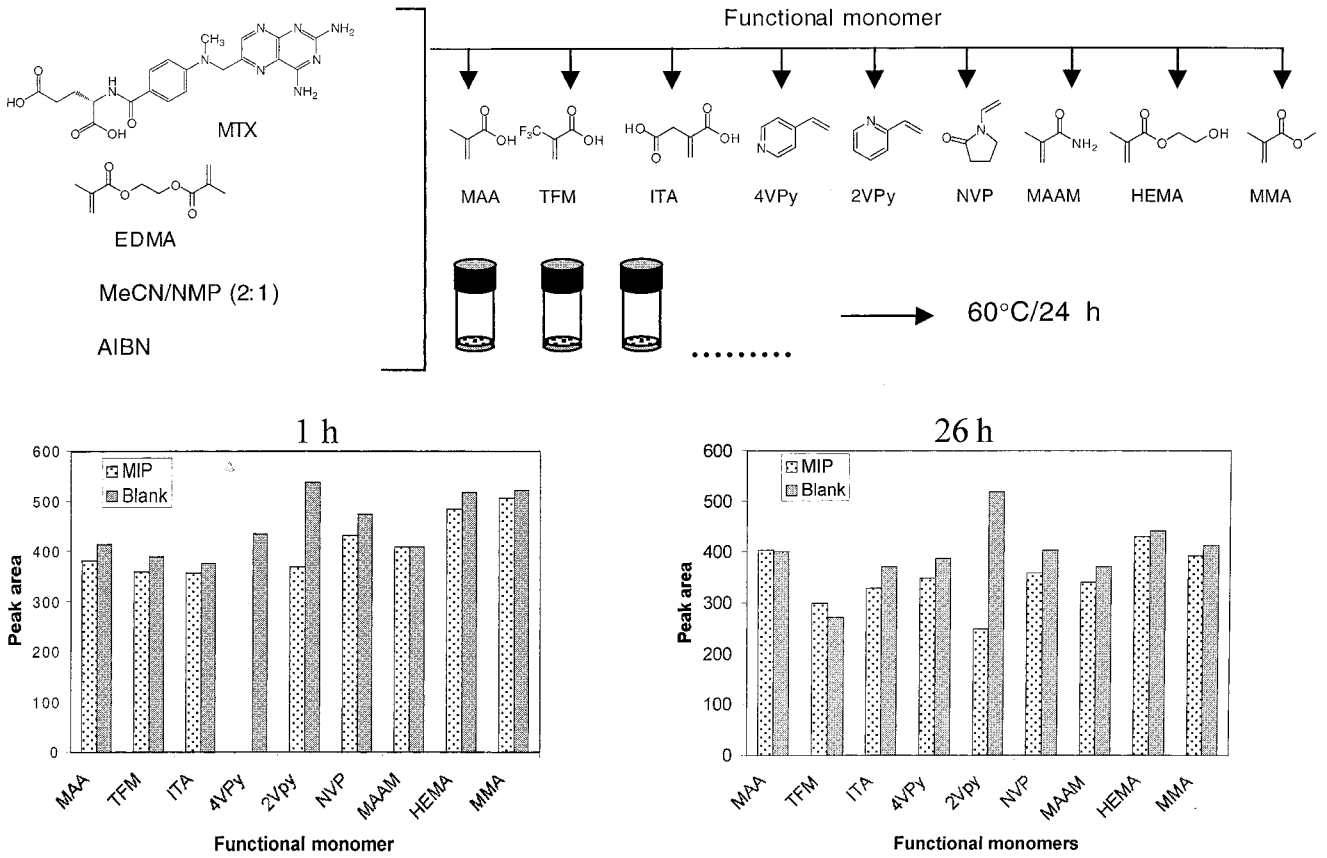

Figure 2. Protocol for the synthesis of miniMIPs using MTX as template and different functional monomers. Below are shown results from the rebinding experiment using the resulting miniMIPs. The graphs show the concentration of template remaining in the supernatant after 1 and 26 h of equilibration of a stock solution of MTX (0.16 mM) in MeCN/NMP (2:1, v/v), respectively.

to be a suitable template due to the potentially strong interactions between the diaminopteridine system and carboxylic acid monomers such as MAA.[29] However, this compound is unstable and poorly soluble in solvents useful for the traditional imprinting procedure (log $P_{OW} = -1.85$).[30] Moreover, its amphoteric character makes it difficult to *a priori* select functional monomers capable of associating with the template in more polar solvents. For instance, the glutamic acid substructure may be best targeted with basic functional monomers (e.g., 2-VPy, 4-VPy), the pteridine and aminobenzoate substructure with acidic functional monomers (e.g., MAA, ITA, TFM), and the aromatic substituents with monomers capable of engaging in aromatic π-electron donor−acceptor interactions (VPy). Such monomers were therefore selected and a series of polymers synthesized on a small scale,[28] followed by evaluation of the template release and rebinding to imprinted and control polymers. In the choice of conditions, a particular difficulty is the appropriate choice of porogen. It is here important to consider factors related to the polymer structure and porosity at the micro-, meso-, and macroscopic levels. This is in addition to the stability and solubility of the monomer−template assemblies, which are of importance for the structure of the binding sites at the nanoscopic level.[31,32] In the molecular recognition by MIPs driven by Brönsted acid−base interactions, it was observed that solvents with poor hydrogen bond capacity resulted in polymers showing efficient recognition, whereas the polarity of the solvent proved to be a less important factor.[32] Thus, the solvent should compete as little as possible with the interaction sites involved in the monomer−template interactions, but at the same time solubilize the complex to prevent precipitation. Binary solvent systems are useful in this regard. Thus, using the polar aprotic solvent acetonitrile (MeCN) as base solvent, MTX could be solubilized by addition of *N*-methylpyrrolidone (NMP). However, the resulting concentration corresponded to only ~5% of that normally used to prepare MIPs.

After synthesis of the miniMIPs, template release was assessed in the porogenic solvent mixture. Only partial release of the template was obtained for some polymers. However, quantitative removal was achieved upon addition of acid. Results from the subsequent rebinding experiment agreed with the results from the release experiment, indicating that the release can be used as a rough predictor for rebinding affinity and selectivity of the MIP.[28] Thus, the MIP prepared using 2-VPy exhibited the largest adsorption and selectivity (Figure 2). This contrasted with the results using MAA as functional monomer, which resulted in a polymer showing no selective adsorption. These results were confirmed in the chromatographic mode when using an upscaled version (see Table 1) of these two polymers as stationary phases (Figure 3). The 2-VPy-MIP exhibited enhanced retention of the template MTX, as well as of the analogues Leu and Fol when compared with the retention on a nonimprinted reference polymer using MeCN/HOAc/H_2O (92.5:5:2.5, v/v/v) as eluent.[33] However, the retention was weak in other mobile phases and the theoretical maximum saturation

(29) For interactions between carboxylic acids and purines and pyrimidines, see: Lancelot, G. *J. Am. Chem. Soc.* **1977**, *99*, 7037.

(30) SRC Physdrop database: http://esc.syrres.com/interkow/PhysProp.htm.

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(33) The separation factor for Fol is uncertain due to the difficulty in determining capacity factors for peaks eluting close to the void marker.

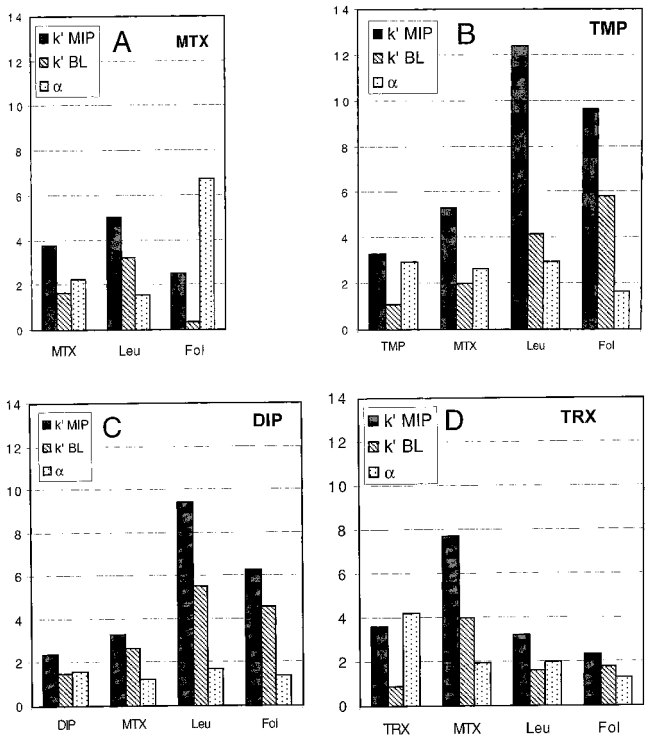


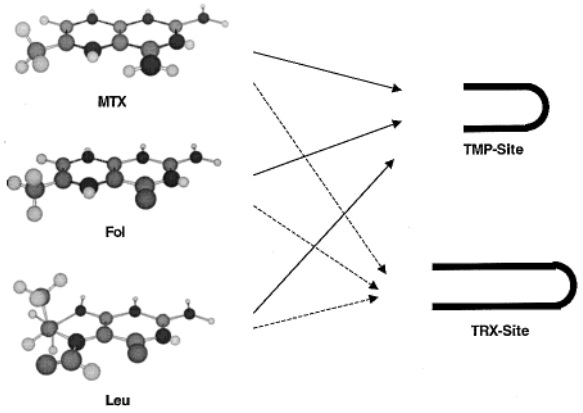
Figure 3. Chromatographic evaluation of columns packed with MIP and blank (BL) polymers. These were (A) PMTX1 and PMTX1BL, (B) PTMP and PTMPBL, (C) PDIP and PDIPBL, and (D) PTRX and PTRXBL. The capacity factors (k') and separation factors (α) graphically estimated from peak maximums of the most retained peaks are shown for the templates and the different folic acid analogues (10 μL of 1 mM solutions).

capacity of the column was low due to the low concentration of template during MIP synthesis. We therefore assessed alternative approaches.

Complementarity toward the Pteridine Substructure. *Chromatographic Retention in Organic Mobile Phases.* A number of lipophilic inhibitors have been developed that exhibit selectivity for microbial DHFR (Chart 1), with trimetrexate being the most potent.[34] Apart from being more lipophilic, these inhibitors are also more basic,[35] leading to strong ionic interactions with the Asp27 function of the enzyme (Chart 2). Assuming that MIP recognition is driven by similar interactions, an interesting question is whether more potent inhibitors are also better templates. Clearly, stronger interactions between the template and the functional monomer will result in a larger portion of the template being complexed prior to polymerization, which will in turn result in a higher yield of templated sites. To test this hypothesis three inhibitors were imprinted using MAA as functional monomer and dichloromethane (DCM) as solvent. At least 90% of the template could be released from the polymers based on elemental analysis, indicating a high accessibility to the templated sites.

The polymers were then evaluated in the chromatographic mode using MeCN/HOAc/H_2O (92.5:5:2.5, v/v/v) as mobile phase (Figure 3). All MIPs exhibited enhanced retention of their templates and of the folic acid analogues. However, they differed

Chart 3



significantly with respect to the selectivity for the folic acid-related compounds. Among the polymers, PTRX showed the strongest and most selective retention of its template, followed by PTMP and PDIP. Interestingly, this agrees with the order of decreasing basicity of the templates[35] and may be the consequence of decreasing monomer−template complexation in the same order (*vide infra*).[36] A higher degree of complexation presumably leads to more selective and a larger number of templated sites.[15] However, selectivity for the template is not directly reflected in the polymers' ability to retain the folic acid derivatives.

In this respect, PTMP showed the strongest retention when compared to the retention on the blank nonimprinted polymer. Thus, Fol, Leu, and MTX were retained with high k' values on the MIP, while considerably less on the blank nonimprinted polymer.[37] This is reflected in the separation factor (α), which was similar for all solutes on this column. On the other hand, using PTRX, the separation factor of the template was more than 2 times higher than those of the folic acid analogues. PDIP showed poorer selectivity, but in aqueous mobile phases (*vide infra*) this polymer also exhibited pronounced selectivity for the folic acid derivatives. This may be due to the hydrophobic substituents of this compound, which introduce an additional hydrophobic driving force in the rebinding to the corresponding cavities in water.[38]

The different retention patterns can be partially explained by considering the shapes of these templates. In Chart 3, MM2 minimized 3D models of template analogues are shown. TMP contains one substituted pyrimidine ring with a conformationally unlocked trimethoxybenzyl group at the 5-position. This may create the space necessary to accommodate the folic acid derivatives, including those containing a nonplanar fused ring system, such as Leu. Indeed, such ring flipping has been observed for TMP bound to the DHFR site.[39] However, TRX, with the fused ring system, will more probably give rise to sites that are complementary toward planar ring systems. The sites templated by TRX are also likely to contain acid groups capable of interacting with the aromatic amino group (*vide infra*) thus leading to a more defined site with poor affinity for the analogues. Leu may not fit into such a site, since the reduced pteridine ring is not planar. This leads to an almost orthogonal orientation of the 6-substituent relative to the ring plane.

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(35) The following pK_a values of the templates and solutes were found or estimated: MTX 3.4, 4.7, 5.7 (a); Leu 3.1, 4.8, 10.4 (a); TMP (N1) 7.1 (b), 6.6 (a); TRX (RNHPh) 5.2 (b, c); TRX (N1) 8.0 (b, d). Sources: (a) *Merck Index*, 11th ed.; Merck & Co, Inc.: Rahway, NJ, 1989. (b) From ref 30, (c) Estimate based on *N*-methyl-3,4,5-trimethoxyaniline (CAS124346-71-0). (d) Estimate based on 2,4-diaminoquinazoline (CAS 1899-48-5).

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(37) A fraction of the solutes eluted early with the void volume on both the MIP and blank columns. The capacity factors refer to the retained fractions.

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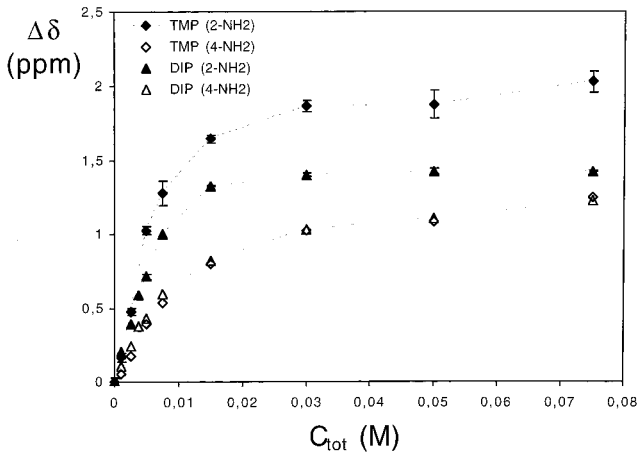


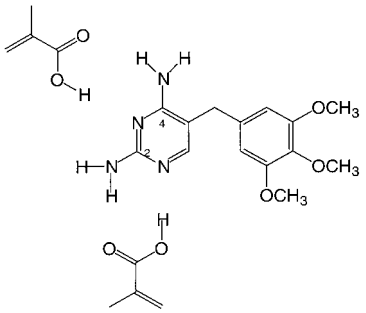
Figure 4. Complexation-induced shifts ($\Delta\delta$) of the amino protons of TMP and DIP (0.005 M) versus total concentration of MAA (C_{tot}) in $CDCl_3$. Each point represents the average of two measurements, and the spread has been indicated when larger than the symbol.

The presence of conformers binding only weakly to the templated sites is another factor that may contribute to weak retention on the MIPs. The most stable conformations of folic acid-related compounds may change with solvent, pH, and temperature.[40] Evidence for the conformational preference of MTX comes from its pH- and temperature-dependent UV spectrum. It is seen that two absorption bands shift to lower wavelengths with decreasing pH or temperature. At low pH, it is known that MTX prefers a folded conformation, allowing intramolecular hydrogen bonds to form between the Glu carboxylic acid groups and the protonated diaminopteridine ring.[40] On the basis of similar spectral shifts, this structure also appeared to be stable in the mobile phase used in Figure 3. A prerequisite for the binding of the folded conformation of MTX to a site complementary toward the pteridine ring structure is that the binding energy exceeds the energy required to destabilize this conformation.

Monomer−Template Association in Solution. To gain further insight into the origin of the selectivity pattern observed for the polymers imprinted with the DHFR inhibitors, the association of the functional monomer and templates was studied in a solution mimicking the prepolymerization mixture. It has been proposed that the structure of the complexes existing in solution prior to polymerization reflects the structure of the subsequently formed binding sites.[15,41−44] This is true only if the complexes remain unchanged during the course of the polymerization and if the resulting sites are not modified due to shrinkage upon removal of the template.[15] The former condition may not apply due to a buildup of strain or a change in the solvent quality with increasing conversion of monomer and the latter due to an insufficient level of cross-linking. Nevertheless, important conclusions may still be drawn from such studies.

Addition of methacrylic acid to a solution of the different inhibitors in $CDCl_3$ resulted in large downfield shifts of both exocyclic amino protons of the templates (Figure 4) and significant shifts of other protons (Figure 5). This is typical for the strong hydrogen bond formation that can be expected between carboxylic acids and N-heterocyclic bases in poorly polar aprotic solvents.[29,45] As proposed for TMP in Chart 4, MAA may form complexes with the templates stabilized by strong cyclic hydrogen bonds at two sites. The most stable of these is expected to involve the more basic N1 nitrogen, leading to the larger shifts of the 2-NH_2 protons compared to the 4-NH_2 protons next to the less basic N3 nitrogen.[35]

Chart 4



Disregarding self-association[46] and assuming a fast exchange between the different states on the NMR time scale, the observed chemical shift (δ_{obs}) corresponds to a mean weighted position between the signals of free solute (template) and the different complexes (SL, LS, SL_2) of the solute (S) with a ligand (L).[47] The complexation induced shift, $\Delta\delta^i$ ($=\delta^i_{obs} - \delta^i_S$), of proton *i* can be expressed as a function of the formation constants (*K*) of the different complexes and the concentration of the free ligand (L). The ratio of the complexation induced shifts for two different protons (*i*, *j*) as a function of [L] is usually constant when only 1:1 complexes are formed whereas for 1:2 complexes the ratio is likely to vary with [L].[47] It is clear from Figure 5 that in the present case the latter situation applies. Thus, the nonparallel plots of the relative complexation-induced shifts ($\Delta\delta/\Delta\delta_{max}$) of the different protons versus the total concentration of MAA (Figure 5A,B) and the one species test for the NH_2 protons (Figure 5C) indicate the presence of higher complexes.[29,48] This plot also shows that the shifts of the 4-NH_2 protons change less with increasing concentration of MAA (smaller slope of the plot in Figure 5A) than those of the 2-NH_2 protons, further supporting a weaker association at this site and agreeing with the expected differences in basicity between the sites (*vide supra*). However, since the concentration of MAA in the MIP was ∼10 times higher than the concentration of MAA corresponding to saturation in the NMR experiment, it is likely that both sites are fully saturated prior to polymerization. It is also striking that the shifts of the 4-NH_2 protons of TMP and DIP are parallel in contrast to those of the 2-NH_2 protons. This indicates that the association at this site is of similar strength in TMP and DIP. Furthermore, the steepness of the plots of the 2-NH_2 shifts in Figures 4 and 5 correlates with the basicity of the compounds,[35] which decreases in the order

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(46) Dilution experiments showed continued downfield shifts of the exocyclic NH_2 protons of DIP and TMP of less than 0.035 ppm when the concentration was increased from 0.00061 to 0.01 M. In view of the observed shifts and corresponding dimerization constant calculated for atrazine in CCl_4,[45] the template dimerization has been neglected in the present system. The dimerization constant of butyric acid in $CDCl_3$ has been computed to 80 M^{-1},[29] suggesting that a significant dimerization of MAA occurs at the concentrations used in the experiment. However, this will lead to an underestimation of the complex formation constants and has therefore been neglected in this qualitative analysis.
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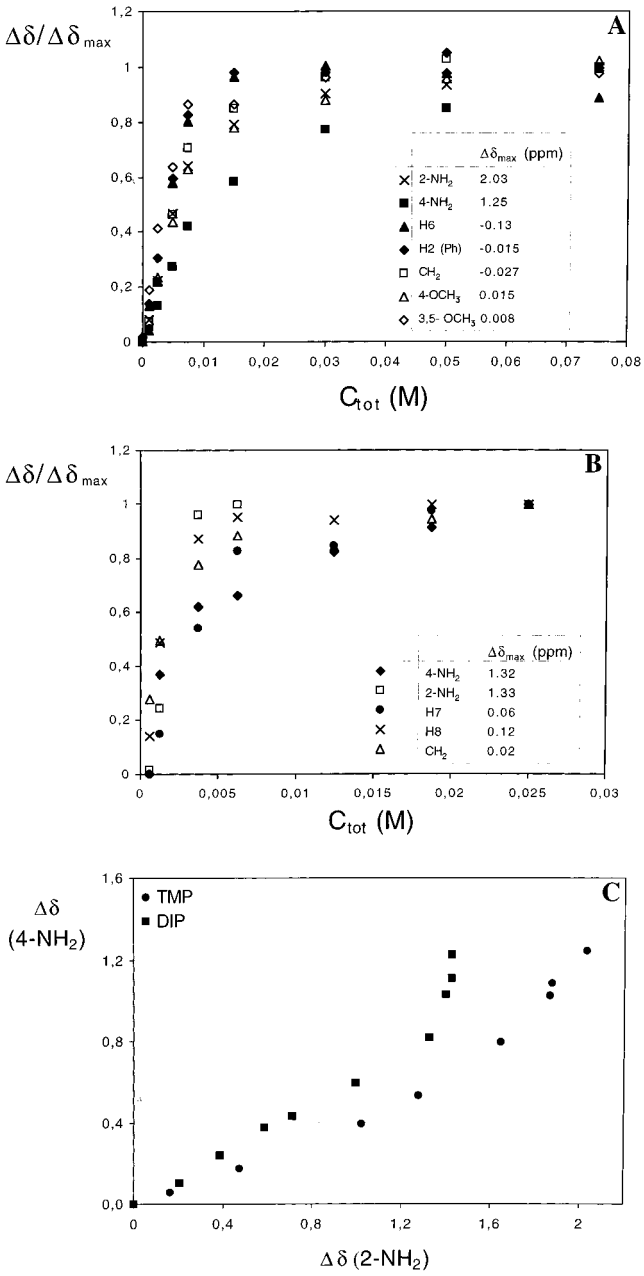

Figure 5. Relative complexation-induced shifts ($\Delta\delta/\Delta\delta_{max}$) for significantly shifted protons of TMP (A) and TRX (B) versus total concentration of MAA (C_{tot}) in $CDCl_3$. The total template concentration was for TMP 0.005 M and for TRX 0.0025 M. (C) Complexation-induced shifts of the 2-NH_2 protons versus those of the 4-NH_2 protons of TMP and DIP. The total concentration of the template and MAA prior to the polymerization were for PTMP and PDIP 0.02 and 0.3 M, respectively, and for PTRX 0.014 and 0.4 M, respectively.

TRX > TMP > DIP. The presence of multimolecular complexes complicates a quantitative evaluation of the titration curves. However, a lower estimate of the formation constants of the most stable 1:1 complexes can be graphically determined as the inverse of the concentration of MAA corresponding to 50% saturation of the 2-NH_2 protons.[47] These values increase in the order DIP 200 M^{-1}, TMP 222 M^{-1}, and TRX 460 M^{-1}, thus also in the order of increasing basicity of the templates.

The plots in Figures 4 and 5 exhibit other interesting features. Several of the protons of TMP, not directly involved in complexation with MAA, are significantly shifted and saturated at lower concentrations than those of the amino groups. The directions of the shifts are different from those of TRX. Thus, while H6 in TMP, adjacent to the primary site of interaction, and the CH_2 group are shifted upfield (−0.13 and −0.027 ppm, respectively), H8 and the CH_2 of TRX are both shifted downfield (0.12 and 0.02 ppm, respectively). The upfield shift may be due to anisotropic shielding caused by torsion of the trimethoxyphenyl group or self-association upon complexation. This may be induced by the stronger charge-transfer interactions that can be expected upon protonation of the pyrimidine ring of TMP, the latter ring being closer to the trimethoxyphenyl group in TMP than in TRX. Finally, the downfield shifts of the CH_2 and NH protons of TRX are in agreement with protonation at the amino group.

Polymer−Template Association: Are the stabilities of the complexes in solution reflected in the binding constants and binding site densities of the resulting MIPs? If this is the case, a quantitative formation of 1:2 complexes between template and monomer would result in a 100% yield of high-affinity templated sites. The question can be answered by performing batch partitioning experiments[15,49−51] or frontal analysis[52,53] providing adsorption isotherms and, in the latter case, additional kinetic information on the binding. In the former case, different amounts of template are added to a mixture containing a fixed amount of polymer. Assuming equilibrium, the concentration of free (C_f) and bound template (n) is determined by quantifying the unbound fraction in the supernatant and the isotherm obtained by plotting the concentration of unbound versus bound species. The isotherms for PTMP/PTMPBL and PDIP/PDIPBL showed that the MIPs adsorbed considerably more template than the blank polymers.[54] The binding isotherm can be fitted with models assuming different distributions of sites of different qualities. For MIPs, the isotherms have been fitted with Langmuir mono ,[55] binary ,[15,50,52,53] or ternary [56,57] site models which assume one, two, or three different classes of uniform sites. The Langmuir binary site model has been shown to be a valid approximation in several cases at higher coverages. However, at low coverages, the isotherms are better fitted with continuous distribution isotherms such as the Freundlich isotherm[52] or using the affinity spectrum approach.[58]

If Langmuir models apply, the association constant K_a and specific site capacity N can be determined from the slope and y intercept, respectively, of lines obtained by least-squares regression of linear regions of the corresponding Scatchard plots (Figure 6). For TMP, these plots are linear except at lower coverages, where curvature is seen, indicating sites of higher affinity. Due to the curvature, attempts to fit these points are necessarily associated with a larger error.[58] The plots for DIP exhibit curvature over the whole concentration interval, and particularly noteworthy are the high-affinity sites present in PDIPBL not seen in PTMPBL (Table 2). This suggests that the

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Table 2. Binding Parameters Determined by Scatchard Analysis of Adsorption Isotherms Obtained from Batch Partitioning Experiments[a]

		MIP		blank		Δ(MIP − blank)[b]	
template	site class	K_a (M^{-1})	N (μmol/g)	K_a (M^{-1})	N (μmol/g)	K_a (M^{-1})	N (μmol/g)
TMP (MeCN)	1	2.7×10^4	27	8.2×10^2	71	4.2×10^4	20
	2	2.6×10^3	116			4.6×10^3	62
TMP ($CHCl_3$)	1	5.2×10^4	29	2.3×10^2	110	6.4×10^4	25
	2	1.2×10^3	130			2.5×10^3	68
DIP (MeCN)	1	2.1×10^5	10	3.3×10^4	7	3.4×10^5	6
	2	2.4×10^3	65	7.3×10^2	69	2.4×10^4	13

[a] The experiment was performed as described in the Experimental Section by equilibrating the polymers (5 mg) in a solution of the template in acetonitrile or chloroform (0.5 mL) for 20 h at room temperature. The theoretical capacity of the MIPs is 51 μmol/g based on the amount added template. The ion-exchange capacity, based on the amount of added MAA, is 872 μmol/g for all polymers. The association constants (K_a) and specific capacity (N) were determined from the slope and y intercept, respectively, of fitted lines obtained by least-squares regression of linear regions of the corresponding Scatchard plots (for PTMP see Figure 6) assuming Langmuir mono or binary site models ($n/C_f = -K_a n + K_a N$). [b] Result from Scatchard plot of the isotherm obtained by subtracting the amount bound to the blank (nonimprinted) polymer (obtained by interpolation) from the amount bound to the MIP.

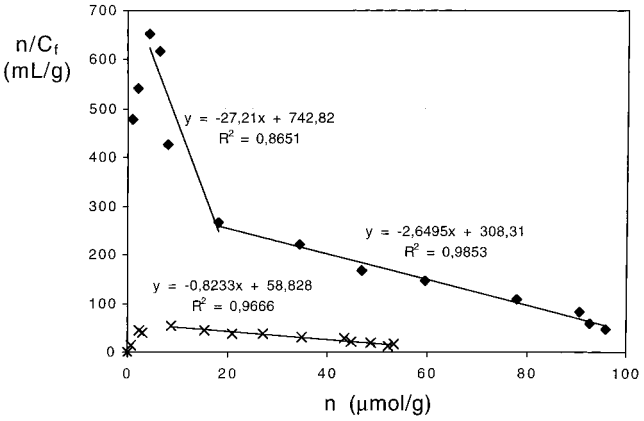

Figure 6. Scatchard plots of the isotherm data for TMP on PTMP (diamonds) and PTMPBL (crosses) in acetonitrile. The association constant (K_a) and specific binding capacity (N) are determined from the slope and y intercept, respectively, of fitted lines ($n/C_f = -K_a n + K_a N$) obtained by least-squares regression (equations and correlation coefficients are shown).

blank polymers possess sites that can strongly interact with DIP but not with TMP. The reason for this is unclear, but it should be noted that DIP contains additional basic sites not present in TMP.

It is interesting to note the difference between the isotherms of TMP and DIP in the same solvent (MeCN). Whereas the isotherms for the blank polymers appear similar in the higher concentration interval ($N = 71$ and 69 μmol/g and $K_a = 820$ and 730 M^{-1} for PTMBBL and PDIPBL, respectively), PTMP exhibits a much larger difference between the imprinted and the blank polymer. This is clearly seen when plotting the difference in adsorbed amount of template to the imprinted and blank polymers at a fixed concentration of free template (Δ(MIP − blank)). For TMP, this levels off at a value of n corresponding to a quantitative yield of templated sites while DIP exhibits the more typical isotherm showing a site yield of ~25%.

Thus, PTMP seems to possess a large class of relatively uniform sites which contrasts with previously described systems using MAA as functional monomer. The following remarks should be made. The yield of templated sites is quantitative for PTMP whereas PDIP exhibits a much lower site yield. This is qualitatively in agreement with results from the NMR titration and the basicity of the molecules (*vide supra*) but probably, other factors also play a role. In view of the weaker nonspecific binding, both site classes observed for PTMP appear to represent templated sites. The parameters determined for PTMP differs somewhat from those obtained from the differential plot, the main difference being the lower K_a's and higher N of the class 2 sites of PTMP. In chloroform, the affinity is higher and the parameters for the class 1 sites for PTMP are close to those calculated for the differential plot. Also, in this case, the main difference lies in the class 2 sites that are obviously diluted with nonspecific sites. In view of the excess amount of MAA used, it is likely that the nonspecific binding can be reduced, without sacrificing the quality of the templated sites, by lowering the concentration of MAA in the monomer mixture.

Chromatographic Retention in Aqueous Mobile Phases. The optimum solvent for rebinding to molecularly imprinted polymers is strongly dependent on the template.[31] Therefore, this has to be carefully optimized in terms of organic/aqueous ratio and pH in order to fully exploit the MIP's ability to recognize a particular guest. A number of studies have shown that MIPs can effectively recognize some guests in aqueous media with equal or higher selectivity to that seen in organic media.[38,57,59,60] This is due to a combination of specific hydrophobic and ionic driving forces acting only in the binding to the MIP, often due to suppression of nonspecific binding by various additives.[60] Figure 7 shows the retention of DIP, Leu, and MTX in mobile phases of different composition and pH using columns packed with PDIP and PDIPBL. The solutes were selectively retained in mobile phases containing either low or high aqueous contents (Figure 7A), thus showing the typical bimodal retention behavior of previously reported MIPs. In aqueous mobile phases with fixed aqueous content, optimization of the pH is another important experiment. DIP was poorly retained, both on the MIP and on the blank at low and high pH, but in the intermediate range a strong retention was seen (Figure 7B**)**. As in previous systems where templates containing Brönsted basic functional groups were used, a maximum retention was observed at an apparent pH near the pK_a value of the solute. Such dependence was previously explained in terms of an ion-exchange process.[61] Thus, multiplication of the charge states of the solutes[35] with that of PDIP indicates whether forces of an attractive or repulsive nature prevail between the polymer and the solute. This simple model adheres well to the experimental data. Particularly interesting is the difference observed between MTX and Leu. Since the latter develops negative charges at a lower pH than MTX, retention decreases more rapidly with increasing pH. Due to this effect, the retention of Leu can be reduced by adjusting the pH to ~5, where MTX is still obviously selectively retained.

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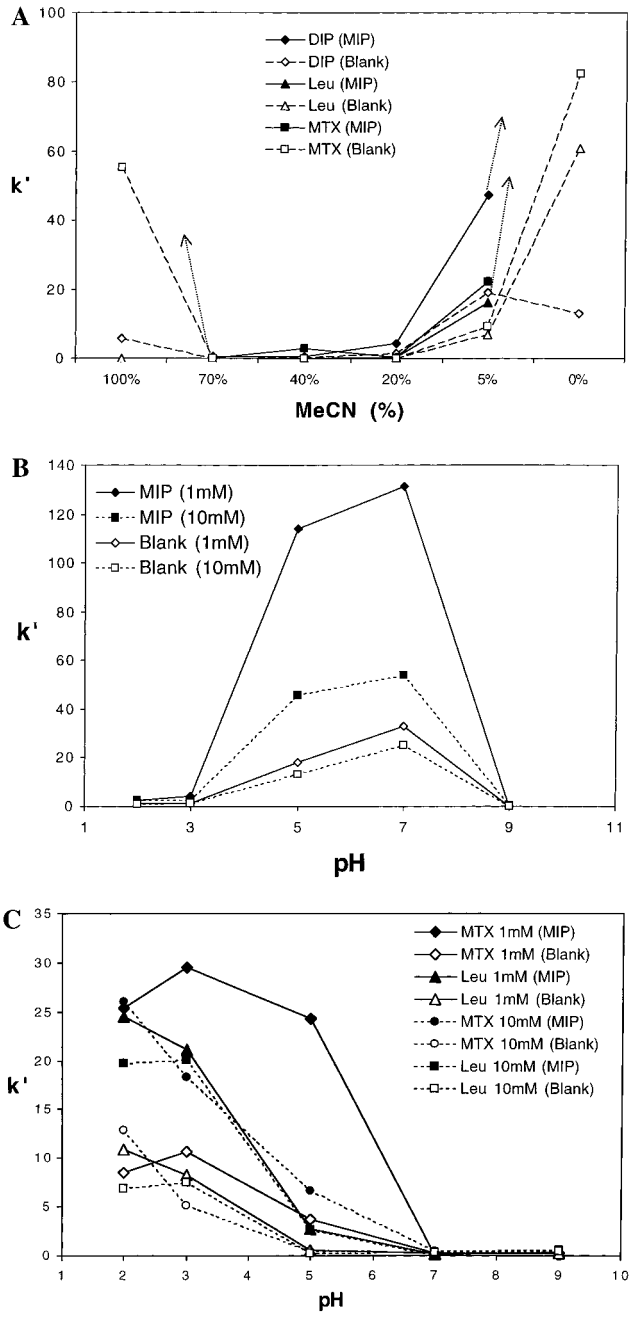

Figure 7. Retention of DIP, Leu, and MTX on columns packed with PDIP (MIP) and PDIPBL (blank) polymers using different mobile-phase compositions: (A) MeCN/(potassium phosphate buffer, 0.02 M, pH 3.0) (*X*/*Y*, v/v); (B) MeCN/(potassium phosphate buffer, 0.02 M, pH *X*) (20:80, v/v); (C) MeCN/(potassium phosphate buffer, 0.02 M, pH *X*) (5:95, v/v). The capacity factors (k') were graphically estimated from the peak maximums of the solutes (10 μL of 1 mM solutions or in (B) and (C) 1 and 10 mM solutions, respectively). In (A), the following retention data for PDIP fell outside the plotted window. DIP and MTX were completely retained in 0 and 100% MeCN within the time measured. Leu was retained with a k' of ~240 in 0% MeCN.

Conclusions

This work has introduced two new concepts to prepare MIPs for complex target molecules. First, the use of MIPs targeted toward substructures of large complex target molecules seems to be a viable approach to overcome the problems related to target solubility, stability, and availability. Second, if the target is known to bind to a biological receptor, the use of simple inhibitors for the same receptor as templates may result in sites capable of selectively binding the target molecule, also in aqueous media. This emphasizes the analogy between the synthetic and biological receptors.

Among the two imprinting approaches assessed to recognize folic acid-related molecules, the use of organic-soluble pteridines as templates appears so far to be the most promising. The resulting materials retained folic acid and analogues with high selectivity. In aqueous media, the chromatographic retention behavior suggested that the recognition was driven by a combination of specific hydrophobic and ionic forces. Another important aspect of the work concerns the general recognition mechanism in MIPs. The correlation between the template basicity and the degree of solution complexation with the selectivity and binding site capacity of the resulting MIPs argues against the previously invoked cluster model.[62] Thus, in the studied concentration regime, the templated sites are most probably monomolecular, containing one or two acid groups positioned for rebinding at the two strongest sites of the guest as depicted in Chart 4.

Important applications of such MIPs can be envisaged. In collaboration with other groups, we are exploring the use of these materials in new improved analytical protocols for the commonly used cytostatic drug MTX.[63] Moreover, the high affinity exhibited by these phases for pteridines is interesting in the light of the newly discovered importance of these compounds as early markers for certain tumors.[64] In a third approach to mimic the DHFR binding site, we are currently exploring the efficacy of novel functional monomers to target the L-glutamic acid substructure.[65]

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Supporting Information Available: ^{1}H NMR complexation-induced shifts for the titration experiments of TMP and DIP in tabular and graphic formats. Adsorption isotherms and Scatchard plots for PDIP, PTMP, and PTRX. UV spectra for MTX and Leu in the different mobile phases and typical elution profiles. This material is available free of charge via the Internet at http://pubs.acs.org.

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