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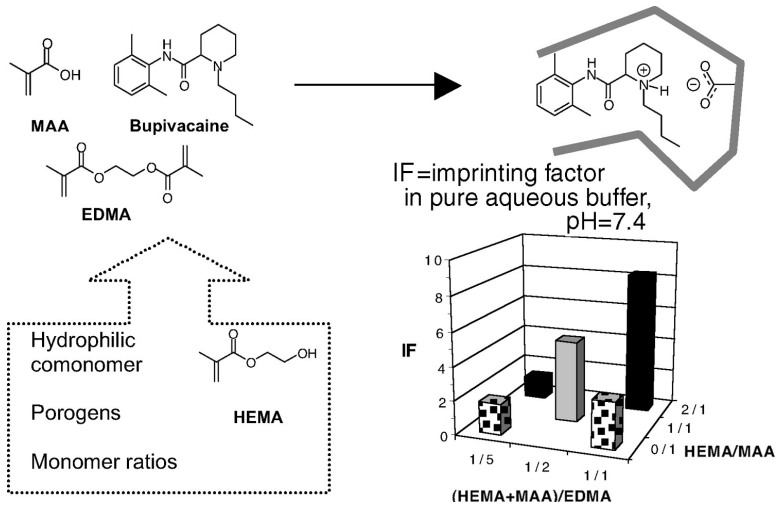

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Water-Compatible Molecularly Imprinted Polymers Obtained via High-Throughput Synthesis and Experimental Design

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Abstract: A technique allowing high-throughput synthesis and evaluation of molecularly imprinted polymer sorbents at a reduced scale (mini-MIPs) was developed and used for the optimization of MIPs for use in pure aqueous environments. The technique incorporated a 4-port liquid-handling robot for the rapid dispensing of monomers, templates, solvents and initiator into the reaction vessels of a 96-well plate. A library of 80 polymers, each ca. 50 mg, could thus be prepared in 24 h. The MIP rebinding capacity and selectivity could be rapidly assessed in the batch mode by quantifying nonbound fractions in parallel using a UV monochromator plate reader. This allowed a complete evaluation of the binding characteristics of an 80 polymer library in approximately 1 week. With the objective of optimizing a polymer imprinted with the local anaesthetic Bupivacaine for use in pure aqueous systems, a polymer library was prepared by varying the original poly(MAA-*co*-EDMA) MIP composition. The variable factors were the added amount of the hydrophilic comonomer, 2-hydroxyethyl methacrylate (HEMA), the cross-linking ratio, and the porogen. This optimization resulted in polymers showing high imprinting factors (IF = K_{MIP}/K_{NIP}) in water as a result, mainly, of reduced binding to the nonimprinted polymer. Normal scale batches of these materials showed strong retention of the template and low nonspecific binding when assessed as chromatographic stationary phases using pure phosphate buffer, pH 7.4, as mobile phase, by equilibrium batch rebinding experiments and as sorbents for extractions of the analyte from blood plasma samples.

Introduction

Molecular imprinting technology is attracting widespread attention due to its potential to deliver robust molecular recognition elements targeted toward essentially any guest present in any environment (e.g. drug enantiomers, hormones, toxins, pesticides, peptides, proteins, and nucleic acids in matrixes ranging from pure organic solvents to biological fluids).[1−3] The previously developed imprinting protocols can be successfully used to produce molecularly imprinted polymers (MIPs) for recognition of a large range of guest molecules predominantly in organic solvent-based media. Although some MIPs synthesized by the use of specifically designed monomer−solvent combinations[4−7] or by the conventional imprinting protocol based on poly(MAA-*co*-EDMA)[8,9] exhibit recognition properties under aqueous conditions, current technology often fails to generate MIPs for use in pure aqueous environments. This is often due to nonspecific hydrophobically driven binding,[9,10] the extent of which depends on the hydrophobicity of the template and the exposed surface of the material. Supressing the nonspecific binding may result in MIPs being closer antibody mimics, which hence can be implemented in separations or chemical sensors in aqueous environments such as biological fluids and environmental waters.

Due to the many parameters influencing the materials' properties at different length scales, as well as the absence of a clear understanding of how these parameters interplay, there are presently no well developed rules to follow for the design of materials exhibiting the desired recognition properties. Thus,

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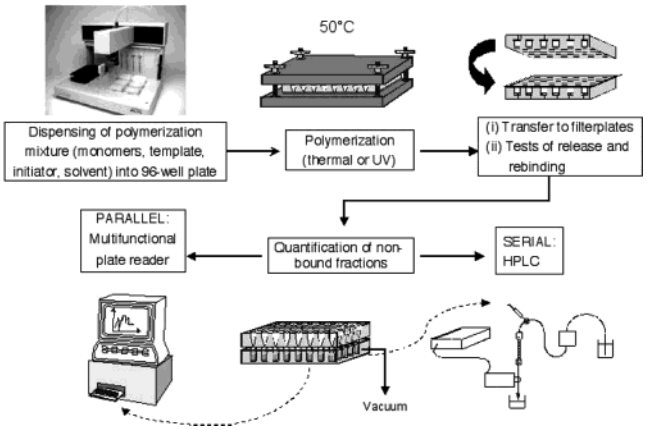

Figure 1. Procedure for high-throughput synthesis and evaluation of large groups of polymers.

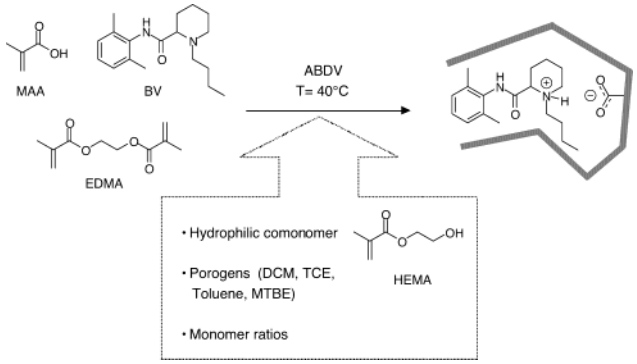

Figure 2. Scheme showing the imprinting of Bupivacaine (BV) in poly(MAA-*co*-EDMA) and factors considered in the synthesis optimization.

combinatorial synthesis approaches, allowing the main factors to be rapidly screened, have offered valuable tools in the development of new MIPs.

We and others recently introduced an in situ synthesis and evaluation technique for MIPs, resulting in libraries of mini-MIPs at the bottom of HPLC-autosampling vials.[11−13] The recognition properties of the polymers could be assessed in situ by HPLC quantification of the nonbound fraction of the template at equilibrium. These techniques were time-consuming due to the slow removal of template and the need for serial analysis of the supernatant solutions. In this report we have cirumvented these problems by the use of filter plates for rapid template removal and a multifunctional plate reader for a parallel analysis of the supernatant fractions (Figure 1).

A complete 96-well plate library can thus be synthesized and evaluated in approximately 1 week, which should be compared with the 3−4× longer time required using the original mini-MIP system. This high-throughput synthesis and screening (HTS) system allows the combinatorial synthesis of large libraries of MIPs with rapid replacement of the liquid phase in the release and rebinding experiments. By using the techniques of experimental design and multivariate analysis,[14] the system constitutes a powerful tool for the rapid optimization of MIPs to attain the desired performance.

Here we have used the HTS system for the optimization of MIPs for use in solid-phase extraction (SPE)[15] targeted toward the local anaesthetic Bupivacaine (Figure 2).

Under aqueous conditions, the hydrophobic surface of these polymers leads to substantial nonspecific binding of the template bupivacaine, as well as nonspecific retention of nonrelated, nonpolar structures.[9,16] In addition, biological sample components, such as proteins and lipids, are strongly adsorbed to the polymer surface. Both processes lead to gradual deterioration of the analytical performance of the extraction and chromatographic columns. In some cases these can be restored by suitable washing schemes, but often, however, the only resort is a frequent change to fresh columns or, alternatively, the use of additional sample pretreatment procedures to remove harmful matrix components.

The ultimate aim was therefore to obtain imprinted sorbents capable of selective extraction of the analyte from pure aqueous buffer with minimum nonspecific binding of the drug as well as other matrix components. This would obviate the need for organic solvent based washing steps. The starting point for the optimization was the well-characterized MIP consisting of poly(MAA-*co*-EDMA) imprinted with Bupivacaine (Figure 2).[16] This polymer was chosen as the reference for subsequent comparisons. The library was constructed by slightly modifying the procedure used to make the reference polymer. The modifications comprised the following: (1) the use of the hydrophilic comonomer 2-hydroxyethyl methacrylate (HEMA), known to impart water compatibility in a number of unrelated systems;[17−20] (2) the use of four porogens (DCM, TCE, toluene, and MTBE), chosen considering health risks, volatility, hydrogen bond capacity, and polarity; (3) the relative ratios of (A) HEMA/MAA and (B) (HEMA + MAA)/EDMA. For each porogen, the latter factors were optimized by a 2^2 factorial design experiment including one center point. The chosen response factors were the partition coefficients of the template on the MIP (K_{MIP}) and on the NIP (K_{NIP}) and the imprinting factor, defined as IF = K_{MIP}/K_{NIP}, the latter reflecting the affinity and concentration of imprinted sites. The best performing polymers were upscaled for assessment as stationary phases by liquid chromatography, by competitive rebinding experiments in aqueous buffers, and as sorbents for extractions of Bupivacaine from blood plasma samples.

Experimental Section

Chemicals. The hydrochloride salts of Bupivacaine (BV), Ropivacaine (RV), and Mepivacaine (MV) were provided by AstraZeneca R&D Södertälje (S-15185 Södertälje, Sweden).

The template (BV) was transformed into the free base as follows: BV HCl (100 mg) was dissolved in water (15 mL). After the pH was adjusted to 11 with Na_2CO_3, Bupivacaine (BV) was extracted into dichloromethane (DCM, 3 × 10 mL). After washing of the organic phase with water (10 mL), it was dried over anhydrous Na_2SO_4 and evaporated to dryness under reduced pressure, yielding the free base quantitatively.

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Table 1. Stock Solutions and Dispensing Scheme for the Preparation of the MiniMIP Library

F1 [(HEMA + MAA)/ EDMA]	F2 (HEMA/ MAA)	MAA (mg)	HEMA (mg)	porogen (μL)	stock soln/mini-MIP (μL)	EDMA/mini-MIP (μL)
1/5	0/1	43	0	400	34	48
1/5	2/1	14	43	400	35	48
1/1	0/1	129	0	400	43	29
1/1	2/1	43	130	400	46	29
1/2	1/1	43	65	400	40	38

Methacrylic acid (MAA), 2-hydroxyethyl methacrylate (HEMA), and ethylene glycol dimethacrylate (EDMA) were purchased from Sigma-Aldrich Chemie GmbH (Taufkirchen, Germany) and purified prior to use as follows: MAA and HEMA were distilled under reduced pressure; EDMA was washed consecutively with 10% NaOH, water, and brine and then dried over $MgSO_4$, filtered, and distilled under reduced pressure. The initiator azo-*N*,*N*′-bis(divaleronitrile) (ABDV) was purchased from Wako Chemicals and used without further purification.

Anhydrous dichloromethane (DCM), anhydrous toluene, methyl *tert*-butyl ether (MTBE), acetonitrile (ACN) for HPLC, methanol (MeOH) for HPLC, water for HPLC, Tween 20, and acetic acid (AcOH) biochemical grade were purchased from Acros (Geel, Belgium). Ethanol came from Kemetyl (Haninge, Sweden). 1,1,1-Trichloroethane (TCE), citric acid, and the buffer salts, Na_2HPO_4, sodium citrate, and CH_3COONH_4, were purchased from Merck (Darmstadt, Germany). All porogens were kept under an argon atmosphere over molecular sieves and were used without further purification. Human albumin (lyophilized) from human serum was purchased from Serva-Heidelberg and tritium-labeled Bupivacaine was obtained from Moravek Biochemicals (Brea, CA). Scintillation cocktail fluid was purchased from Wallac (Turkku, Finland). The water used in the HPLC study was obtained from a Milli-Q unit equipped with a Quantum VX ultrapure V-lonex cartridge from Millipore.

Apparatus. The 96-well PTFE microtiter plate and PTFE coated closures were obtained from Radleys (Shire Hill, Saffron Walden, Essex, U.K.). The chemically resistant 96 filter- and microtiter plates were a gift from Whatman Polyfiltronics (Maidstone, Kent, U.K.).

The 96 microtiter glass plates and PTFE-coated silicon Septa were obtained from Zinsser Analytic (Frankfurt, Germany).

Quartz-glass microtiter plates were obtained from Hellma Worldwide (Müllheim, Germany).

All chromatographic evaluations were performed using a Hewlett-Packard instrument (HP 1050) equipped with a quaternary pump, an autosampler, a diode array detector, and an HP workstation. The parallel UV measurements were performed using a multifunctional plate reader SAFIRE, from Tecan Deutschland GmbH (Crailsheim, Germany). For pipetting of the polymer solutions a 4-port liquid sample handler LISSY from Zinsser Analytic (Frankfurt, Germany), equipped with Zinsser WinLissy software, was used.

The pipetting of washing solutions and the preparation of monomer solutions was performed with Eppendorf Research Pro 8-manifold pipets (Eppendorf AG, Hamburg, Germany).

The nitrogen sorption study was performed on a Quantachrome Nova 2000 (Quantachrome Corp., Boynton Beach, FL).

The equipment used for the equilibrium rebinding and competitive rebinding experiments included an Eppendorff centrifuge from Hettich (Tuttlingen, Germany) and a WinSpectral 1414 scintillation counter from Wallac (Turku, Finland).

Mini-MIP Library. (a) Synthesis. For each of the four porogens (DCM, TCE, toluene, MTBE), two initiator solutions (with and without template) were prepared by mixing ABDV (24 mg) with 640 μL of the porogen. For the MIP series, BV (19.4 mg) was added as template. Five different stock solutions of the functional monomers were prepared per porogen as specified in Table 1. Prior to preparation of the solutions the porogens, EDMA, MAA, and HEMA were purged with argon for 2 min.

The initiator solution (40 μL with or without template) was then dispensed into the 96-well PTFE microtiter plate, followed by addition of the functional monomer stock solutions and the cross-linker EDMA (see Table 1).

Prior to the polymerization, the microtiter plate was sealed with a PTFE-coated silicon septum. Each pipetting step was accompanied by degassing with argon for 5 s.

The microtiter plate was sealed with Viton rings and a PTFE cover plate and then heated in oven for 24 h at 50 °C.

(b) Template Release, Extraction, and Rebinding Experiments. After polymerization the polymers were transferred to a 96-well filter plate. The template was extracted by successive washing steps with 600 μL of MeOH/AcOH/H_2O (60/30/10, v/v/v) until the template could no longer be detected in the washing solution. This was followed by a conditioning step with methanol. Prior to the rebinding experiments, the library was subjected to a final wash with the same solvent as used in the rebinding step.

The rebinding experiments were then performed by adding 600 μL of a solution of BV (1 mM) in ACN (HPLC grade) or BV as its HCl salt (1 mM) in NaH_2PO_4/Na_2HPO_4 buffer solution (HPLC grade) (25 mM, pH 7.4) respectively to each well of the microtiter plate.

The concentration of free BV was determined after 16 h by direct absorbance reading using a multifunctional plate reader or by sequential HPLC analysis. In both cases the following procedure was followed: After addition of the incubation solutions the filter plate was sealed on top and bottom with PTFE-coated silicon closures with the aid of a press. After the incubation of the polymers the closures were removed and the solutions were sucked through undervacuum into a microtiter plate from which samples were taken for the subsequent measurements. For the evaluation using the plate reader, 200 μL samples from each well were transferred into a 96-well quartz plate and measured at 230 nm. For the HPLC evaluation 10 μL samples were injected and analyzed using a C18 reversed-phase column (EU Material UMMA 049S, 5 μm, 150 × 4 mm), with the mobile phase ACN/10 mM ammonium acetate buffer (pH 4.5) (70/30, v/v), UV detection at 230 nm, and the BV/BV HCl rebinding solution as external standard. After each rebinding experiment the polymers were reconditioned by first washing with MeOH/AcOH/H_2O (60/30/10, v/v/v) until template could no longer be detected, followed by a wash with the rebinding solvent.

Normal Scale Batches. Upscaled versions of the polymer pairs (imprinted and nonimprinted) 9 and 14, as well as the reference polymer pair 11, were prepared as follows. MAA (340 μL, 4 mmol), HEMA (MIP/NIP 9 and 14, 970 μL, 8 mmol; MIP/NIP 11, no HEMA), EDMA (MIP/NIP 9 and 14, 2.5 mL, 12 mmol; MIP/NIP 11, 3.8 mL, 20 mmol), ABDV (MIP/NIP 9 and 14, 60 mg, 0.24 mmol; MIP/NIP 11, 110 mg, 0.44 mmol), and BV (all MIPs: 97 mg, 0.33 mmol) were dissolved in the porogen (5.6 mL of TCE (MIP/NIP 9); 5.6 mL of toluene (MIP/NIP 14); 5.2 mL of toluene (MIP/NIP 11)) and then transferred to glass polymerization tubes (14 mm i.d.). Each solution was then degassed with nitrogen for 5 min, and the tubes were sealed and heated at 40 °C for 24 h. After the polymerization, the tubes were smashed and the polymer monolith was coarsely ground and then extracted with MeOH for 24 h in a Soxhlet apparatus. Thereafter the particles were further crushed first with a mortar and pestle and then in a ball-mill, sieved under water, and dried at 40 °C.

Chromatographic Evaluations. The particles (25−50 μm size fraction) were slurried in MeOH/water (80/20, v/v) and packed into HPLC columns (30 mm × 4 mm) at a maximum pressure of 200 bar using a compressed gas-driven slurry packer and MeOH/water (80/20, v/v) as pushing solvent. Thereafter the polymers were tested by comparing the retention factors (k) for BV injected as 10 μL of 10 mM solutions of BV dissolved in ACN or BV HCl dissolved in water on the different polymers in different mobile phases. The retention factors were calculated from the estimated retention times (t) of the

peak maxima and the elution time of the void marker (t_0) acetone or MeOH as $k = (t - t_0)/t_0$. The UV detection wavelength was 230 nm and the flow rate 1.0 mL/min.

Swelling Tests. The polymer volume swelling was estimated using volume-calibrated NMR tubes filled with 0.5 mL of well-packed polymer particles (25−50 μm size fraction). After addition of solvent (ACN or water), the tubes were allowed to stand at room temperature until no further change of the swollen bed volume was observed. The swelling was determined as the ratio of the swollen bed volume to the dry bed volume.

Equilibrium Rebinding and Competitive Rebinding Experiments. Prior to both the equilibrium rebinding and the competitive rebinding experiments, a 20 mg/ mL suspension of polymer particles (25−50 μm size fraction) was prepared and left for 24 h. Before the equilibrium rebinding experiments, 1 mL of incubation solutions was prepared in Eppendorf tubes. These contained 400 μL of citrate buffer (125 mM, pH 5), ethanol (5%), Tween 20 (0.05%), and radiolabeled Bupivacaine (50 μL, ca. 1.2 ng) (30 000−50 000 dpm) as well as 0, 0.25, 0.5, 1.0, 2.5, 5, or 10 mg of polymer added from the above stock suspension. The final volume was adjusted to 1 mL with water. The Eppendorf tubes were placed on a rocking bed for 16 h to allow equilibration. After 16 h the tubes were removed and centrifuged at 18 000 rpm for 5 min and 0.5 mL of supernatant was removed. To the supernatant, 5 mL of scintillation fluid was added, and scintillation counting of the free radiolabeled Bupivacaine was performed.

For the competitive rebinding experiments, 1 mL of incubation solutions was prepared in Eppendorf vials. These contained 400 μL of citrate buffer (125 mM, pH 5), ethanol (5%), Tween 20 (0.05%), and radiolabeled Bupivacaine (50 μL, ca. 1.2 ng) (30 000−50 000 dpm). The amount of polymer added to the Eppendorf tubes was equivalent to the PC_{50} values calculated from the equilibrium rebinding experiments. These values were 1.3 mg/mL for MIP 11, 5.0 mg/mL for NIP 11, 4.3 mg/mL for MIP 9. and 7.2 mg/mL for the MIP 14. Finally, a competing analyte, Bupivacaine, Ropivacaine, or Mepivacaine, was added at varying concentrations between 1 and 330 000 nM. As previously described for the equilibrium rebinding experiments, the Eppendorf tubes were placed on a rocking bed for 16 h and centrifuged and the supernatant was counted after addition of 5 mL of scintillation cocktail.

Protein Binding. Adsorption of human serum albumin on polymers 9, 11, and 14 was tested using the same columns as used in the chromatographic evaluations (vide supra). Before use, the columns were preconditioned by successive washings with water, HCl (0.07 M), water, MeOH/water (50/50), water, and finally 25 mM phosphate buffer (pH 7.4). The adsorption test was performed in 25 mM phosphate buffer at pH 7.4 at a flow rate of 1 mL/min with a UV detector recording at a wavelength of 290 nm. A human serum albumin solution (50 mg/mL) made up in the mobile phase buffer was injected (100 μL) four times consecutively. The amount of adsorbed protein after each injection was calculated from the area of the breakthrough peak in relation to the area of the peak obtained after injection of the protein solution in absence of a column.

Solid-Phase Extraction. Two standard polypropylene SPE cartridges containing each 24 mg of polymer were prepared for each polymer. Prior to use, they were conditioned by washing with 1 mL of methanol followed by 1 mL of water. The plasma sample was prepared as follows. To a plasma sample (400 μL) was added nonlabeled Bupivacaine (1000 nM) and 100 000 DPM (approximately 10 nM) of labeled Bupivacaine, ethylcaine (8 μM) in water (100 μL), and citrate buffer (0.4 M, pH 5, containing 0.1% Tween 20) (500 μL). This sample was then applied at the top of the column. This was followed by a wash step with 2 × 1 mL water, one wash step with 0.5 mL of acetonitrile and two elution steps with 2 × 1 mL of 92% acetonitrile, 6% water, and 2% TEA. To 0.1 mL of each fraction was added 5 mL of scintillation fluid followed by scintillation counting.

Results and Discussion

Choice of Factors and Experimental Design. The starting point for the optimization was the previously reported MIP of the poly(MAA-*co*-EDMA)-type targeted toward Bupivacaine (Figure 2). As with the majority of MIPs described in the literature, this material is of the macroreticulate type prepared by free radical polymerization in the presence of high levels of cross-linking monomers and a porogenic solvent.[21] This gives rise to amorphous materials containing nanometer-sized binding sites in addition to larger sized pores. In this one-step approach, the successful imprinting of a particular template depends on a simultaneous fulfillment of several criteria. First, molecular binding sites for the template and the target molecule(s) need to be generated at or near the pore walls. Second, the surface of the material must be compatible with the medium of application. Most MIPs prepared by the self-assembly approach contain a methacrylate- or styrene-based polymer backbone which imparts a respectively slight or pronounced hydrophobic character to the material.[22] While these materials commonly exhibit pronounced recognition in low dielectric strength media, a hydrophobically driven nonspecific adsorption is observed when they are used in water. On the other hand, the surface of hydrophilic materials (e.g. poly(acrylamides), poly(HEMA)) are wetted by water and exhibit low nonspecific adsorption in such media.

Typically, the generation of stable high affinity imprinted sites requires the following:[21]

(i) one or more functional monomers capable of forming stable complexes with the template molecule during polymerization; (ii) a high nominal cross-linking level as lower levels (<50%) are insufficient for preserving the templated sites for longer periods of time; (iii) the use of an aprotic apolar solvent as porogen as this favors the electrostatic interactions most commonly utilized between the functional monomers and the template.

These requirements are to some extent contradictory to the approaches available to incorporate hydrophilic surface properties:

(1) Polar porogens[22] can be used. These solvate the polar functional groups of the monomers leaving them exposed at the pore walls after porogen removal. This in turn leads to reduction of hydrophobic nonspecific binding.

(2) Hydrophilic comonomers (e.g. HEMA, acrylamide) or cross-linkers (e.g. pentaerythritoltriacrylate, methylenebis(acrylamide)) can be used in the imprinting step.[5−7,17−20] Depending on the polarity of the porogen these will be more or less exposed at the pore walls of the materials.

(3) Postgrafting of hydrophilic chains can be employed.[23]

In view of the recent reports on imprinted HEMA-based hydrogels,[17] we decided to investigate approach 2 by addition of HEMA under conventional imprinting conditions satisfying the criteria for binding site stabilization. Thus, terpolymers of the type poly(MAA-HEMA-EDMA) were prepared in the presence of four different aprotic porogens. Due to the complex-

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Table 2. Monomer Compositions Used To Prepare the Polymer Library and Results from the Rebinding Tests of Bupivacaine-Imprinted and Nonimprinted Polymers in Acetonitrile[a]

		F1	F2	HPLC			reader		
MIP/NIP	porogen	[(HEM A + MAA)/EDMA]	(HEMA/MAA)	K_{MIP} (mL/g)	K_{NIP} (mL/g)	IF	K_{MIP} (mL/g)	K_{NIP} (mL/g)	IF
1	DCM	1/5	0/1	49.8 ± 3.2	21.3 ± 1.9	2.3	50.7 ± 3.3	22.0 ± 1.3	2.3
2		1/5	2/1	10.5 ± 0.6	6.1 ± 0.1	1.7	11.2 ± 0.5	6.3 ± 0.0	1.8
3		1/1	0/1	281 ± 21	161 ± 0.8	1.7	272 ± 19	143 ± 1.5	1.9
4		1/1	2/1	37.2 ± 0.3	26.4 ± 1.1	1.4	49.7 ± 5.7	27.1 ± 0.9	1.8
5		1/2	1/1	61.1 ± 2.5	26.7 ± 0.3	2.3	58.9 ± 2.4	26.3 ± 0.8	2.2
6	TCE	1/5	0/1	58.0	13.3 ± 0.0	4.4	61.4	15.3 ± 0.5	4.0
7		1/5	2/1	7.5 ± 0.0	3.6 ± 0.1	2.1	11.6 ± 1.6	3.6 ± 0.2	3.3
8		1/1	0/1	83.5	12.3 ± 0.7	6.8	31.1	12.8 ± 0.9	2.4
9		1/1	2/1	14.7 ± 0.3	0.8 ± 0.0	20	15.3 ± 0.2	0.5 ± 0.1	33
10		1/2	1/1	49.6 ± 0.3	5.9 ± 0.0	8.4	40.1 ± 0.7	6.0 ± 0.2	6.6
11	toluene	1/5	0/1	59.8 ± 0.5	14.6 ± 0.5	4.1	60.1 ± 0.0	13.3 ± 0.2	4.5
12		1/5	2/1	7.6 ± 0.1	4.0 ± 0.3	1.9	8.5 ± 0.0	3.8 ± 0.1	2.3
13		1/1	0/1	34.3 ± 0.4	9.1 ± 0.2	3.8	33.0 ± 37	9.6 ± 0.2	3.4
14		1/1	2/1	4.9 ± 0.3	0.2 ± 0.0	21	2.3 ± 0.6	0.2 ± 0.0	9.8
15		1/2	1/1	31.1 ± 0.5	5.9 ± 0.1	5.2	31.1 ± 1.2	6.0 ± 0.0	5.2
16	MTBE	1/5	0/1	49.6 ± 0.3	17.1 ± 0.3	2.9	48.8 ± 0.0	18.3 ± 0.2	2.7
17		1/5	2/1	9.1	5.1 ± 1.0	1.8	9.4	5.2 ± 0.6	1.8
18		1/1	0/1	204 ± 40	203	1.0	173 ± 29	170	1.0
19		1/1	2/1	23.3 ± 0.1	14.8	1.6	21.9 ± 0.1	14.4	1.5
20		1/2	1/1	28.5 ± 1.1	18.4 ± 0.0	1.6	33.1 ± 3.3	17.6 ± 0.2	1.9

[a] The polymer library was prepared as described in the Experimental Section by addition of a total of 300 μmol of monomer, 4 μmol of BV, and 120 μL of porogen containing the initiator ABDV (1.5 mg) per well. The molar ratios of functional monomers to EDMA and HEMA to MAA are given as F1 and F2, respectively. After degassing and sealing, the plates were left at 50 °C for 24 h. After exhaustive extraction of the template, the rebinding test was performed by incubating each polymer with a 1 mM solution of BV in acetonitrile for 16 h. Quantification of the free BV was performed by HPLC or reader analysis of the supernatant fractions. From these values and the weight of each polymer, the partition coefficients (K) and the imprinting factor IF ($=K_{MIP}/K_{NIP}$) were calculated. Errors are given as the spread between the two replicas. No error limits are given for the members lacking replicas.

ity of the imprinting process (vide supra), the polymer composition was optimized by following the theory of experimental design. The continuous factors chosen were the molar ratio of the functional monomers to the cross-linking monomer (F1: (HEMA + MAA)/EDMA) and the molar ratio of the functional monomers (F2: HEMA/MAA). Finally, the type of porogen was chosen as a discontinuous factor (F3: DCM, toluene, TCE, MTBE). The experiment was performed according to a 2^2 full factorial design with one center point and the limits of F1 being 1/5 and 1/1, whereas F2 was varied within the interval 0/1 and 2/1. These intervals were chosen taking the above criteria for the stabilization of the monomer−template complexes and the imprinted sites into consideration. It should be noted that the lower values of F1 and F2 correspond to the conditions used to prepare the reference MIP that was previously extensively investigated as a solid-phase extraction sorbent and in competitive assays.[16] The choice of porogens was guided by previous reports, as well as by their chemical and physical properties. DCM and toluene are the most commonly employed of the poorly polar porogens and have been used to generate good binding sites for a large number of low molecular templates. 1,1,1-Trichloroethane (TCE) is similar to DCM in terms of polarity ($\epsilon_{DCM} = 8.93$, $\epsilon_{TCE} = 7.24$) but exhibits attractive properties in terms of its lower toxicity and higher boiling point ($bp_{DCM} = 40$ °C; $bp_{TCE} = 75$ °C), the latter being of particular importance when polymerizing thermally in non-pressure-proof reaction chambers. Finally, MTBE, in combination with MMA/EDMA (1/4), has been shown to generate high surface area materials comparable to or grater than those prepared using toluene, acetonitrile, or chlorobenzene.[24]

Mini-MIP Library for Bupivacaine Recognition. The composition of the polymerization mixtures of each member of the library is seen in Tables 1 and 2. These were prepared by the use of a pipetting robot, by pipetting degassed stock solutions of the monomers, initiator, template, and porogen to the wells of a PTFE 96-well microtiter plate covered with a silicone rubber sealing mat. Each pipetting step was accompanied by 5 s degassing with argon. An 80-polymer library was prepared consisting of 20 different monomer compositions, one replica/member, and an equal number of nonimprinted control polymers. The polymerization was performed by heating the plate, sealed tightly with a PTFE lid fixed in place by the aid of a press, for 24 h in an oven. After polymerization, the wells were visually inspected for the presence of liquid, unreacted monomer, and solid polymer. For five of the members (see Table 2) no polymer was obtained, presumably due to clogging of the needle due to septum disintegration. After drying of the polymers, they were weighed and transferred mechanically to deep-well filter plates. The weights of the polymers, measured after the first rebinding experiment, were found to be between 40 and 55 mg, indicating a high conversion of the monomers.

To remove the template, the polymer library was then subjected to an exhaustive extraction by repeated additions of MeOH/AcOH/H_2O (60/30/10, v/v/v) whereafter the polymers were conditioned by incubating them with MeOH followed by MeCN/H_2O (70/30 (v/v)). After drying, the ability of the polymers to bind the template was assessed by adding 500 μL of a Bupivacaine solution (1 mM) in MeCN to each well, followed by incubation for 16 h.

The nonbound fractions were then quantified in parallel using a multifunction plate reader or in series by HPLC (triplicate injections). In the former method, the supernatants were directly vacuum transferred to a receiver plate followed by transfer to a 96-well quartz plate. The supernatant concentrations could then be directly determined from the absorbance of each well at 230

(24) Santora, B. P.; Gagné, M. R.; Moloy, K. G.; Radu, N. S. *Macromolecules* **2001**, *34*, 658−661.

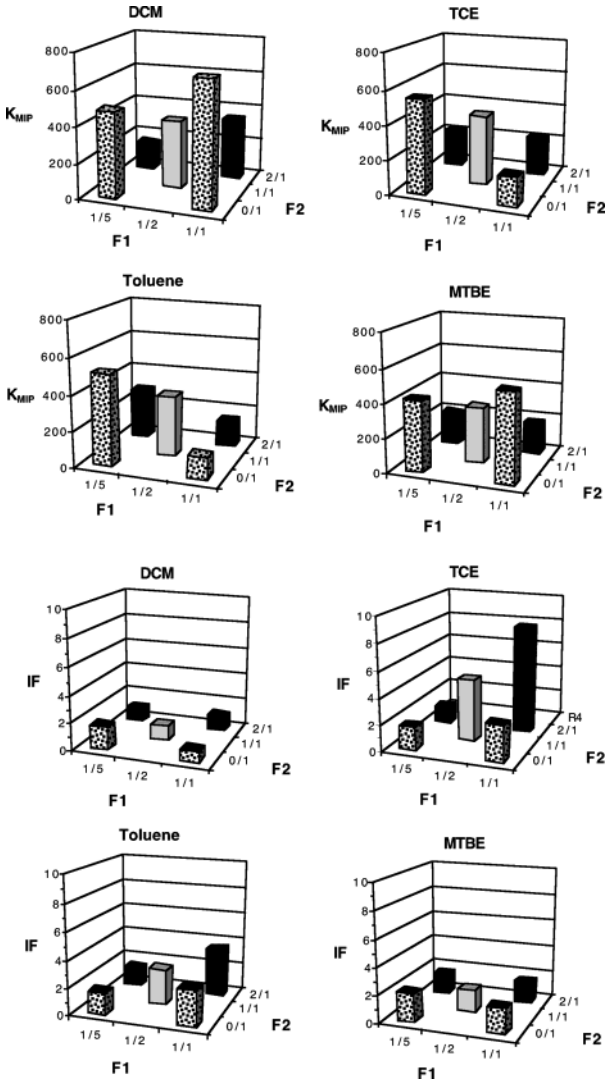

Figure 3. 3D representation of the results from the rebinding of BV−HCl in NaH_2PO_4/Na_2HPO_4 buffer solution (HPLC grade) (25 mM, pH 7.4) to the polymer library showing in (A) the partition coefficients for the imprinted polymers (K_{MIP}) and in (B) the corresponding imprinting factors (IF) calculated from the supernatant free concentrations of BV (1 mM) after binding to the polymer library. Conditions are otherwise as described in Table 2.

nm. Table 2 shows the average partition coefficients and imprinting factors from the two replicas using both quantification techniques.

The small difference between the values of the replicas indicates that the polymers can be reproducibly prepared in the well plate format.[25] Also gratifying is the agreement between the results obtained using the two quantification techniques. This implies that reliable quantification of the nonbound fractions of a 96-well plate library can be obtained using parallel reading in a fraction (1−2 min) of the time required using serial HPLC analysis (ca. 45 h). The above results appear particularly promising in view of the weak chromophore of the template.

The partition coefficients (K) decreased with an increasing HEMA/MAA ratio (F2). This was common for the four groups of polymers prepared using the different porogens and is likely due to a lower concentration of the more strongly interacting functional monomer, methacrylic acid, in the preparations. Otherwise, the group of polymers prepared using TCE as porogen showed trends similar to the one prepared using toluene, with a decrease in K with increasing (HEMA + MAA)/EDMA (F1), whereas the opposite was observed for the other two groups prepared using DCM and MTBE as porogens. This groupwise behavior is also reflected in the imprinting factors (IF). Here the DCM and MTBE groups all exhibit IFs between 2 and 3 whereas the TCE and toluene groups, as a whole, showed higher IFs, with the highest values (IF $\approx$ 20) observed for the polymers 9 and 14 prepared using high ratios of HEMA/MAA and (HEMA + MAA)/EDMA, F2 and F1. This is due to a strong decrease in the binding to the corresponding nonimprinted polymers.[26]

(25) A repetition of the rebinding experiment after wash and conditioning of the materials gave similar results.

Table 3. Composition of Monomer Mixtures Used To Prepare the Upscaled Polymers, Selected from the Mini-MIP Library, and Structural Data for the Polymers Obtained from Nitrogen Sorption Measurements[a]

polymer	porogen	F1 [(HEMA + MAA)/EDMA]	F2 (HEMA/MAA)	S^b (m^2/g)	$V_p{}^b$ (mL/g)
MIP 9	TCE	1/1	2/1	95	0.21
NIP 9	TCE	1/1	2/1	35	0.080
MIP 14	toluene	1/1	2/1	49	0.088
NIP 14	toluene	1/1	2/1	15	0.023
MIP 11	toluene	1/5	0/1	297	0.62
NIP 11	toluene	1/5	0/1	308	0.68

[a] The polymers were prepared and characterized as described in the Experimental Section. [b] Results from nitrogen sorption isotherms calculated using the BJH method applied to the desorption branch of the isotherm. S = cumulative surface area, and V_p = the total volume of pores with diameter less than 50 nm.

Encouraged by these results, we repeated the rebinding experiments in pure aqueous buffer. The rebinding results are best viewed in 3D diagrams (Figure 3) with F1 and F2 on the x and z axis and the response factors (K and IF) on the y axis. As can be seen in Figure 3, the results were qualitatively similar to the results in Table 2 with the highest IF again seen for the polymer prepared using TCE (MIP 9) followed by toluene (MIP 14) as porogens and the highest values of F1 and F2. This indicates that imprinted polymers exhibiting dramatically reduced nonspecific binding in pure aqueous buffers can be prepared from specific monomer and porogen compositions. To further investigate the most promising members of the library polymers 9 and 14 were prepared in larger scale together with polymers 11 used as a reference, prepared similarly to the previously investigated MIP (Table 3).

Characterization of Upscaled Batches. Polymerization and workup were carried out by following a well-established procedure.[27] Particles of the 25−50 μm size fraction were isolated and packed in stainless steel columns, and chromatographic tests were thereafter performed using first acetonitrile and then potassium phosphate buffer, pH 7.4 as mobile phases. Figure 4 shows the elution profiles obtained from 100 nmol injections of BV (in MeCN) or BV−HCl (in buffer) on the imprinted and nonimprinted polymers 14 and 11.

In pure acetonitrile the reference MIP 11 strongly retains the solute with a retention factor (k) of 11, whereas retention on

(26) As remarked by one reviewer, the groupwise behavior correlates with the volatility of the porogens. Thus, the poorer performance of the DCM and MTBE materials could be ascribed to partial evaporation of the porogen during polymerization. However, ligand binding results for normal scale batches corresponding to polymers 4 and 10 agreed with those of the well plate.

(27) Sellergren, B.; Shea, K. J. *J. Chromatogr.* **1993**, *635*, 31.

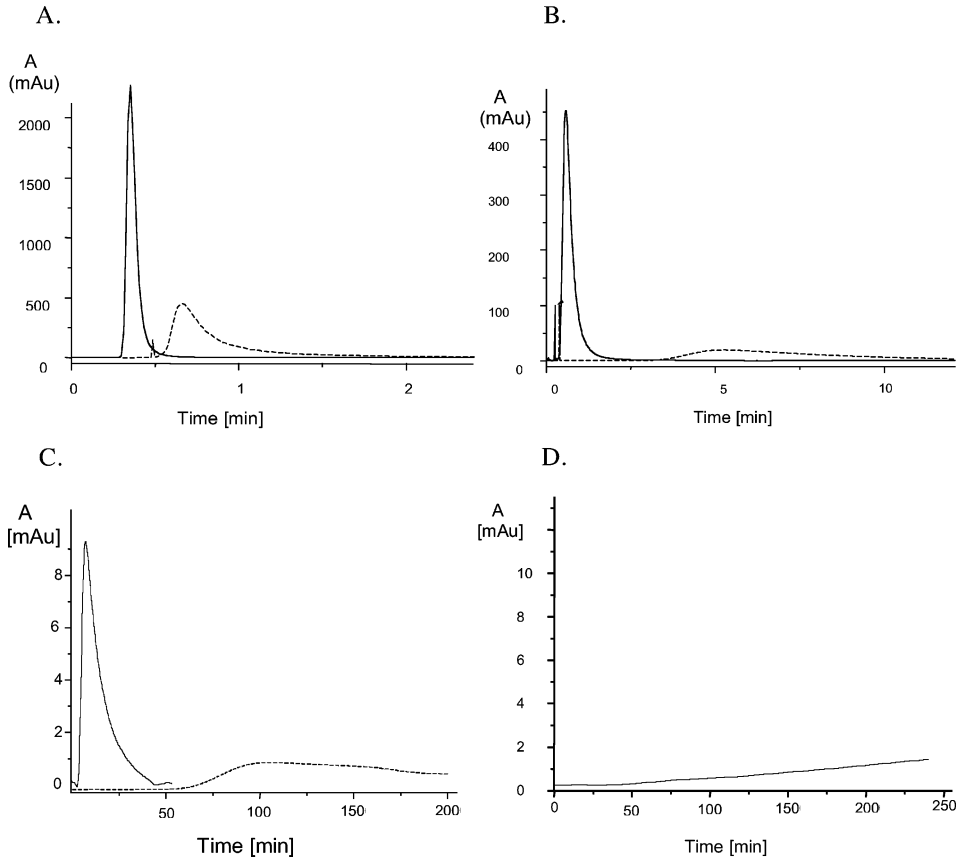

Figure 4. Comparison of the elution profiles of BV (100 nmol) obtained using polymers 14 (A, C) and polymers 11 (B, D) (MIP, dashed lines; NIP, solid lines) in acetonitrile (A, B) or NaH_2PO_4/Na_2HPO_4 buffer solution (HPLC grade) (25 mM, pH 7.4) (C, D).

the nonimprinted polymer is weak ($k = 1.25$) (Figure 4B). This contrasts with the elution profiles on polymers 14, where the solute elutes below 1 min on both the MIP ($k = 0.37$) and the NIP ($k = 0.017$) (Figure 4A).[28] Despite this apparently poor performance this polymer exhibited high imprinting factor in pure aqueous buffer (Figure 4C). Thus, the solute elutes with a peak maximum at ca. 10 min on the NIP while the peak maximum on the MIP, despite the characteristically broad peak, can be estimated at ca.100 min.[28] As seen in Figure 4D, the solute is strongly retained on the reference MIP and NIP under these conditions. These observations confirm the results obtained from the polymer library investigation and indicate that the optimized polymers exhibit considerably lower nonspecific binding in pure aqueous media compared to the reference polymer.

The selectivity of the polymers was subsequently investigated by ligand binding experiments[29] in the batch mode using radiolabeled Bupivacaine. To relate the data to previous studies using the reference polymer 11,[9] an optimized buffer composition consisting of citrate buffer (pH 5) containing ethanol (5%) and detergent (Tween 20, 0.05%) was used. The adsorption of [^{3}H]-labeled BV was first studied as a function of the concentration of added polymer (Figure 5). The isotherms essentially confirm the results obtained using the polymers as chromatographic stationary phases.

Table 4. IC_{50} Values for Bupivacaine (BV), Mepivacaine (MV), and Ropivacaine (RV) on the BV MIPs and the Corresponding Relative IC_{50} Values[a]

	IC_{50} (μM)			IC_{50}/IC_{50} (BV)		
MIP	BV	RV	MV	BV	RV	MV
11	1.6	44	388	1	27	242
9	41	1429	1777	1	35	43
14	65	140	7427	1	2.2	113

[a] Samples of polymers 11, 9, and 14 (1, 4, and 6 mg, respectively) were incubated with solutions (1 mL) each containing 50 mM citrate buffer, pH 5, 5% ethanol, 0.05% Tween 20, approximately 1.2 ng of radiolabeled Bupivacaine, and different concentrations between 1 and 330 000 nM of Bupivacaine (BV), Mepivacaine (MV), or Ropivacaine (RV). After incubation for 16 h, the supernatant was analyzed by scintillation counting. The IC_{50} values correspond to the concentration of the competing ligand (BV, RV, or MV) displacing 50% of bound radiolabeled BV.

Thus, the NIPs of polymers 9 and 14 exhibit low or nonexistent affinity for the solute, whereas the reference NIP 11 shows a steep increase in bound BV as a function of polymer concentration. The MIPs, on the other hand, all bind the solute under these conditions, although the isotherms exhibit different shapes. This is reflected in the polymer concentration (PC_{50}) required to adsorb 50% of radiolabeled BV. Whereas only 1.3 mg of MIP 11 is needed to adsorb 50% of the solute, the corresponding values for MIP 9 and MIP 14 are 4.3 and 7.2 mg, respectively. Thus, the supression of the nonspecific binding seems to have compromised the average affinity for the template. One possible explanation for this can be found by studying the structure and porosity of the materials. As expected from their lower cross-linking level, the water-compatible materials exhibit a lower surface area and pore volume compared to the

(28) The retention factors obtained in acetonitrile using MIP and NIP 9 were 0.66 and 0.054, respectively. In pure aqueous buffer BV eluted at 32 min on NIP 9 and after more than 75 min on MIP 9.
(29) Sellergren, B.; Andersson, L. I. *Methods Enzymol.* **2000**, *22*, 92−106.

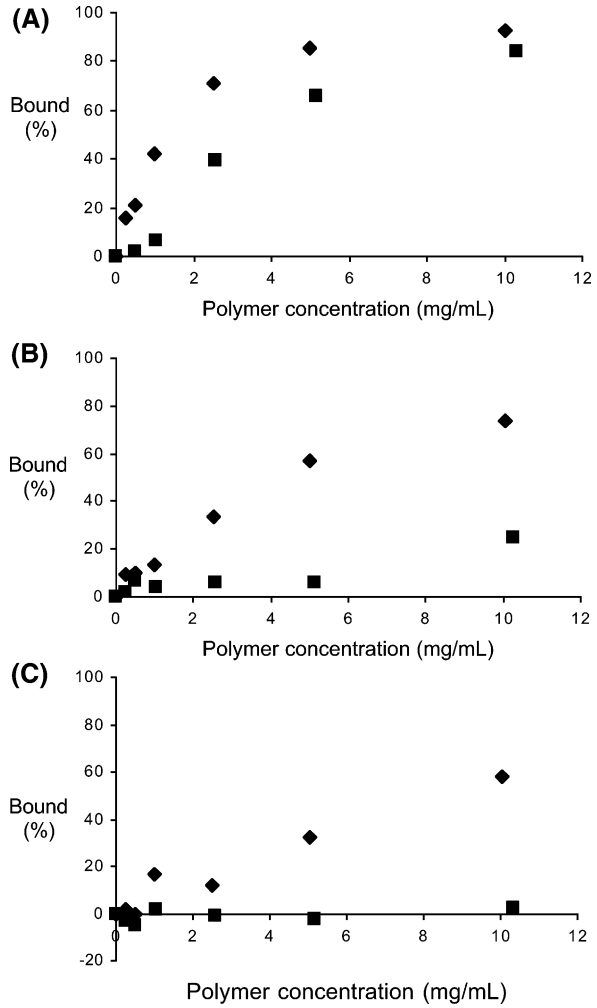

Figure 5. Fraction of bound BV in % to imprinted (diamonds) and nonimprinted (squares) polymers as a function of the amount of added polymers 11 (A), 9 (B) and 14 (C) to a 1 mL solution of BV (aproximately 1.2 ng, 30 000−50 000 dpm) in 50 mM citrate buffer (pH 5) containing ethanol (5%) and detergent (Tween 20, 0.05%).

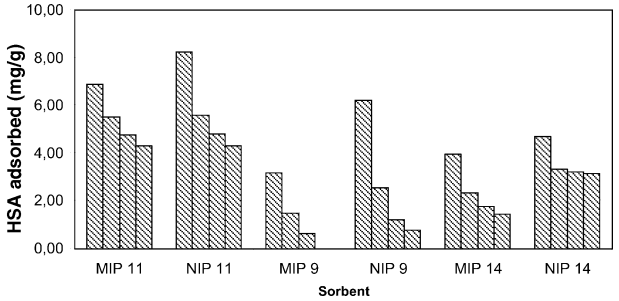

Figure 6. Amount of human serum albumin (HSA) adsorbed per gram of dry polymer sorbent in sodium phosphate buffer (25mM) at pH 7.4 after four consecutive injections (100 μL) of a standard HSA solution (50 mg/mL). The cumulative protein adsorptions were the following: MIP, 11/21 mg/g; NIP, 11/23 mg/g; MIP, 9/5 mg/g; NIP, 9/11 mg/g; MIP, 14/10 mg/g; NIP, 14/14 mg/g. The conditions were otherwise as described in the Experimental Section.

conventional polymers. This by itself can contribute to the more shallow slope of their adsorption isotherms. More alarming are the differencies in surface areas and pore volumes between the MIPs and NIPs of the watercompatible materials. The NIPs exhibit ca. 3 times lower values than corresponding MIPs whereas, for the reference materials 11, the values are similar. However, the stronger swelling of the NIPs and the similar

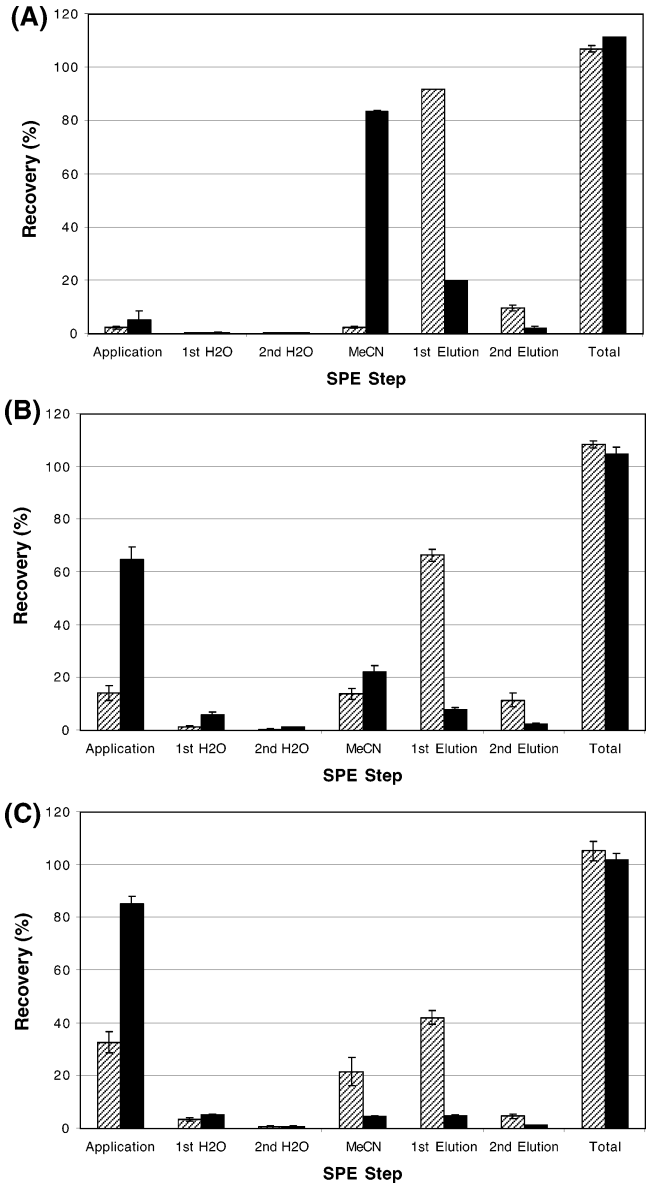

Figure 7. Recoveries of BV in each fraction obtained after solid-phase extraction of BV spiked blood plasma (1000 nM) samples of MIPs (striped bars) and NIPs (solid bars) using polymers 11 (A), 9 (B), and 14 (C) as sorbents. The sorbents (25 mg each) were packed in polypropylene SPE cartridges and subjected to the conditioning and extraction protocol described in the Experimental Section. After application of the plasma sample (1 mL), the columns were washed with 2 × 1 mL water and one time with 0.5 mL of acetonitrile followed by two elution steps with 1 mL of 92% acetonitrile, 6% water, and 2% TEA.

elution times measured for the void markers indicate that the polymers are less different regarding their swollen state morphology.[30] This highlights nevertheless the problems associated with the choice of appropriate control polymers to estimate imprinting effects. In this case, supporting evidence for the presence of templated sites is needed from selectivity assessments. We therefore performed competitive binding experiments by challenging the MIP and NIP with structurally similar compounds (Table 4).

(30) The swelling measured in acetonitrile was for MIP 9/1.19 (mL/mL) and for NIP 9/1.25 (mL/mL). The elution volume of the void marker acetonitrile after subtraction of the extracolumn volume was 13% larger for MIP 14 than for NIP 14 and 18% larger for MIP 9 than for NIP 9.

In this experiment a fixed amount of polymer is added to a dilute solution of [^{3}H]-BV resulting in uptake of ca. 50% of the radiolabeled BV. Incremental amounts of competing ligands (BV, RV, MV) are subsequently added. The IC_{50} values correspond to the concentration of the competitive ligands required to displace 50% of bound radiolabeled BV. The lower affinity of the water-compatible polymers is further confirmed by the more than 20 times higher IC_{50} values for unlabeled BV on these polymers compared to the reference material. Nevertheless, considering the much higher IC_{50} values of the two structural analogues RV and MV on both MIP 9 and MIP 14 and the corresponding relative IC_{50} values, they exhibit selectivities on a par with the reference material MIP 11.

To prove the usefulness of the hydrophilized materials, they were tested as sorbents for direct solid-phase extraction of BV from blood plasma samples and compared with the reference materials MIP/NIP 11. Prior to the test, nonspecific adsorption of plasma proteins on the different sorbents was estimated by injecting 100 μL of a standard human serum albumin (HSA) solution (50 mg/mL) on the columns used in Figure 4. According to the results in Figure 6, polymers 11 show the lowest recovery of protein followed by polymers 14 and 9. This agrees with the expected order of decreasing hydrophobicity where polymers 11 should possess the most hydrophobic character implied by the results in Figure 5. This also implies that the surfaces of polymers 14 and 9 are likely to be less susceptible to fouling by plasma proteins.

Next, plasma samples spiked with a known amount of labeled BV (1000 nM) was applied on the different sorbents packed in solid phase extraction (SPE) cartridges. After a simple wash protocol the analyte was eluted (Figure 7). In Figure 7 the recoveries in each step expressed as an average of at least 3 extractions using two replicate columns are given. A pronounced difference in the recovery profiles can be seen. Whereas the reference MIP and NIP number 11 adsorb BV nonspecifically and almost quantitatively in the application step, polymers 9 and 14 exhibit a pronounced difference between the MIP and NIP in the same step. This difference is most pronounced for polymers 9 with more than 60% of the applied BV breaking through on the NIP whereas only ca. 15% broke through on the MIP. In agreement with the results shown in Figure 5, binding of BV to polymers 9 seems also to be overall stronger than to polymers 14.

Conclusions

High-throughput synthesis and evaluation of polymer libraries in a 96-well plate format allows rapid optimization and fine-tuning of the molecular recognition properties of molecularly imprinted polymers. This tool was successfully used to find conditions for MIP synthesis leading to reduced nonspecific binding in fully aqueous environments. Thus, MIPs selective for the local anaesthetic bupivacaine in pure aqueous buffers could be prepared. Despite the lower binding affinity of these MIPs, they exhibit high selectivity and apparently low nonspecific binding in water. This proved useful for direct and selective extraction of Bupivacaine from blood plasma samples and should prove useful as well for other biological matrixes. Furthermore, applications of such MIPs as receptor layers in chemical sensors aimed at direct determinations of analytes in aqueous samples should be feasible.

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