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Synthetic Human Serum Albumin Sudlow I Binding Site Mimics

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Here, we report the design, synthesis, and characterization of molecularly imprinted polymer (MIP) derived mimics of the human serum albumin (HSA) Sudlow I site—the binding site for the anticoagulant warfarin. MIP design was based upon a combination of experimental (^{1}H NMR) and computational (molecular dynamics) methods. Two MIPs and corresponding nonimprinted reference polymers were synthesized and characterized (scanning electron microscopy; nitrogen sorption; and Fourier transform infrared spectroscopy). MIP−ligand recognition was examined using radioligand binding studies, where the largest number of selective sites was found in a warfarin-imprinted methacrylic acid−ethylene dimethacrylate copolymer (MAA-MIP). The warfarin selectivity of this MIP was confirmed using radioligand displacement and zonal chromatographic studies. A direct comparison of MIP−warfarin binding characteristics with those of the HSA Sudlow I binding site was made, and similarities in site population (per gram polymer or protein) and affinities were observed. The warfarin selectivity of the MIP suggests its potential for use as a recognition element in a MIP-based warfarin sensor and even as a model to aid in understanding and steering blood−plasma protein-regulated transport processes or even for the development of warfarin sensors.

Introduction

Warfarin is an oral anticoagulant extensively used in the treatment of thrombolic disorders.[1] Because of a narrow therapeutic window and the influence of factors such as food intake, metabolic rates, and drug−drug interactions on its anticoagulant effect, the warfarin dosage is highly patient dependent. Another key factor influencing warfarin bioavailability is its transport in blood, where some 99% is found bound to the Sudlow binding sites of human serum albumin (HSA[a]).[2] Currently, the anticoagulant effect of warfarin is indirectly measured through the correlation of the clotting time (prothrombin time) and the amount of the drug present in blood.[3] Because of the difficulties in maintaining patient coagulation response within the therapeutic window, regular monitoring is often required. To improve warfarin therapy, the development of alternative measuring methods, ideally both more robust and more sensitive, for the direct determination of warfarin is highly desirable.[4,5] Ultimately, such methods may offer the possibility for patient self-monitoring.

The inherent instability of biomolecule-based sensors has prompted the use of biomimetic materials as alternative recognition elements for use in various sensor detection platforms.[6−10] Molecularly imprinted polymers (MIPs) offer much promise in this regard. Molecular imprinting has been used to develop synthetic polymers with selectivity for a wide range of molecular structures, in particular small molecules,[11−13] although even for biological macromolecular systems.[14−19] Various (bio)sensing elements have been developed using MIPs, in part motivated by the generally low production cost and the physical and chemical robustness of these materials.[11,20,21] It was envisaged that polymers with selectivity for warfarin may ultimately prove useful in the development of new analytical approaches for the detection of this important pharmaceutical. Here, we present the first example of a MIP with warfarin selectivity and draw parallels between polymer−ligand recognition and the situation when binding to the Sudlow I site of HSA.[22] This binding site is comprised of a series of hydrophobic domains in combination with several functionalities providing electrostatic interactions to warfarin. Moreover, this study includes the first example of the use of full polymerization system−system comparisons for identifying suitable polymer compositions. The potential for using such a polymer as a recognition element in a warfarin sensor is discussed.

Results and Discussion

Despite its widespread use and the difficulties in adapting dosage to clinical function, no functional assay for the direct detection of the anticoagulant warfarin in blood is currently used in general clinical practice. A significant factor underlying the difficulties associated with its determination is warfarin's inherent structural diversity, an issue that has impact on both the bioavailability of the drug and its determination in blood. Warfarin's capacity to undergo environment-dependent isomerism was also expected to impinge upon the outcome of its use as a template in molecular imprinting protocols.

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[a] Abbreviations: 4VP, 4-vinyl pyridine; AcOH, acetic acid; AIBN, 2, 2′-azobis-(2-isobutyronitrile); BET, Brunauer, Emmett, and Teller; BJH, Barret, Joyner, and Halenda; EC_{50}, effective concentration needed for 50% displacement; EDMA, ethylene dimethacrylate; FT-IR, Fourier transform infrared; HPLC, high-performance liquid chromatography; HSA, human serum albumin; IF, imprinting factor; K_D, equilibrium dissociation constant; MAA, methacrylic acid; MD, molecular dynamics; MIP, molecularly imprinted polymer; NOESY, nuclear Overhauser effect spectroscopy; REF, reference polymer; SEM, scanning electron microscopy; TLC, thin-layer chromatography.

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Table 1. Polymer Physical Characterization

physical properties	MAA-MIP	MAA-REF	4VP-MIP	4VP-REF
BET surface area ($m^2\ g^{-1}$)	46.0 ± 0.6	119.6 ± 1.0	2.8 ± 0.3	2.3 ± 0.2
BJH micropore volume ($cm^3\ g^{-1}$)	0.0057	0.0186	0.0002	0.0003

The molecular imprinting technique[23] provides access to synthetic polymers with predetermined recognition characteristics, for example, a memory for a given ion or molecular structure. Current molecular imprinting dogma dictates that it is the stability and nature of monomer−template prepolymerization complexes that determine the recognition properties of a MIP. In support of this, the underlying interactions have been examined using a range of spectroscopic techniques, NMR,[12,24−26] UV spectroscopy,[27] and even theoretical approaches.[6,24,28−31] Recently, we have presented a strategy for elucidating the mechanisms underlying a MIP−polymerization system, where statistical analysis of molecular dynamics studies (MD) is used in conjunction with spectroscopic studies.[24,28] Herein, we have utilized this approach, whereby a series of ^{1}H NMR titration experiments and MD simulations were employed for system design (choice between functional monomers). In MD studies, modeling accounted for the warfarin isomeric distribution found in corresponding solvent mixtures as previously reported by Valente and co-workers[32] (Supporting Information, Tables S2 and S3 for conditions used for MD simulations), where the ratio of the three dominant isomers (major and minor hemiketals and open forms; 45:40:15) was represented in calculations, studies that have been verified and expanded upon using a series of two-dimensional ^{1}H correlation spectroscopy (COSY) and nuclear Overhauser effect spectroscopy (NOESY) studies (Supporting Information, Figures S1−S3). These computational and spectroscopic techniques were used to determine the extent and nature of the interactions between the warfarin and the two functional monomers selected for use in this study [methacrylic acid (MAA) and 4-vinylpyridine]; for NMR studies, the monomer analogues were used, acetic-acid-d_4 and pyridine-d_5, respectively. These two monomers were considered to provide characteristics representative of the polar amino acid residues present in the Sudlow I site. Together with the relatively nonpolar cross-linker used in this study, ethylene dimethacrylate (EDMA), these copolymers were envisaged to provide warfarin binding environments reminiscent of those found in HSA. From the initial investigations, results indicated similar binding strengths (K_D = 0.3−0.4 M, Supporting Information, Table S1) for complex formation between the functional monomer analogues studied and warfarin. Moreover, results obtained from a continuous variation study revealed the presence of MAA−warfarin complexes of a 1:1 stoichiometry based on changes in the chemical shifts of protons adjacent to the −OH functionality of the major cyclic hemiketal (Supporting Information, Figure S5). Even though complex binding strengths could successfully be estimated using a series of ^{1}H NMR titration experiments, the absence of protons near potential interaction points on warfarin suggested that MD studies would provide a more detailed characterization of complex formation. Subsequent evaluation of atomic grid density data generated from the MD trajectories of monomer around warfarin (Supporting Information, Figure S6−S7) revealed high densities for the basic monomer 4-vinyl pyridine (4VP) near the hydroxyl group of warfarin, thus indicating the formation of a strong interaction that is ionic in character. Although present at lower densities at this functionality, the acidic monomer acetic acid (AcOH) was also observed near the coumarin carbonyl group, suggesting the presence of hydrogen bonds to both of these positions on warfarin. Collectively, the theoretical and spectroscopic studies indicated that the carboxylic acid functionality present in MAA provides two significant modes of interaction with template.

MAA− and 4VP−EDMA copolymers were synthesized using low-temperature UV-initiated polymerization in chloroform, in either the presence (MIP) or the absence [reference polymer (REF)] of the template, racemic warfarin, the form used in the clinic. The resultant bulk polymers were crushed, sieved, and washed to remove residual template using a previously described protocol employing a series of pH and polarity jumps.[33] Polymer particles were characterized using Fourier transform infrared (FT-IR) spectroscopy to verify the incorporation of the anticipated functionalities in the resultant polymer and successful template removal (Supporting Information, Figures S8 and S9). Scanning electron microscopy (SEM) studies provided insight concerning the distribution of polymer particle sizes (Supporting Information, Figure S10).

Physical characterization of the polymers revealed significant differences in surface areas (Table 1). In the case of the MAA polymers, a ~2.5-fold difference in surface area was found between the MAA-MIP and the MAA-REF where the larger surface area of the MAA-REF was suggested to be related to the greater porosity of this material. This was found to be consistent with results obtained with similar systems employing other templates.[34] The corresponding values for the 4VP polymers show that these polymers were notably less porous than the MAA polymers. Moreover, the surface areas and porosities of the imprinted and reference 4VP polymers were similar.

To evaluate the warfarin rebinding capacity, the polymers were investigated in a series of radioligand polymer titration studies (Figure 1).[33,35] In subsequent analysis of the data, the binding capacities of the polymers were normalized with respect to their surface areas. Importantly, the normalized binding of warfarin to the MAA-MIP was found to be higher than the corresponding binding capacity of the MAA-REF. In the case of the 4VP polymers, although both MIP and REF demonstrated higher normalized binding capacities than the MAA polymers, the higher binding to the 4VP-REF than the 4VP-MIP suggested the absence of an imprinting effect in the these polymers. Collectively, it may be suggested that the difference observed in warfarin binding to the 4VP polymers is due to high degree of nonspecific binding. On the basis of the absence of pores in this material, binding occurs to the surface of the polymers. Moreover, this hypothesis is supported by the fact that this rebinding is based only on a single electrostatic interaction point, as supported by the results of the full system MD prepolymerization mixtures studies. Accordingly, the large difference in the warfarin binding capacity of the MAA polymers led us to determine that a large number of specific binding sites are present in the MAA-MIP matrix. Furthermore, the Brunauer, Emmett, and Teller (BET) analysis in conjunction with the obtained binding data indicate the presence of pores in the MAA-MIP, which are important for warfarin rebinding to imprints located inside the polymer matrix.

The warfarin selectivity of the MAA-MIP was investigated through a series of radioligand displacement studies in which

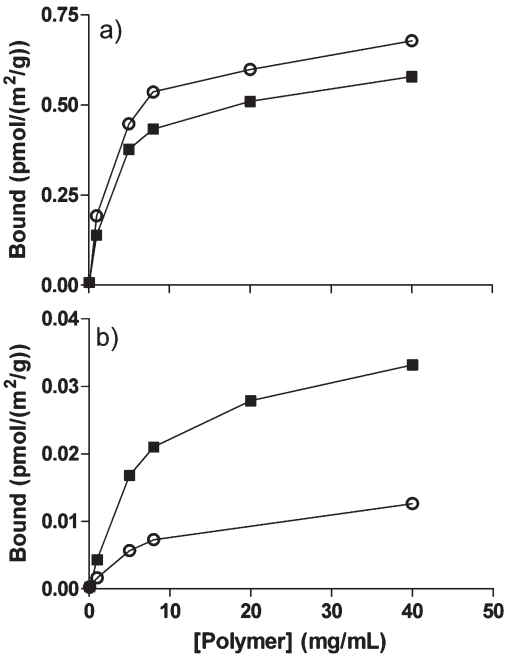

Figure 1. Polymer titrations in toluene at 293 K: (a) 4VP and (b) MAA polymers. Two picomoles of warfarin was added to different concentrations of MIP (■) or REF (○). The amount of warfarin bound to the polymers was scaled using BET surface area data, presented in Table 1.

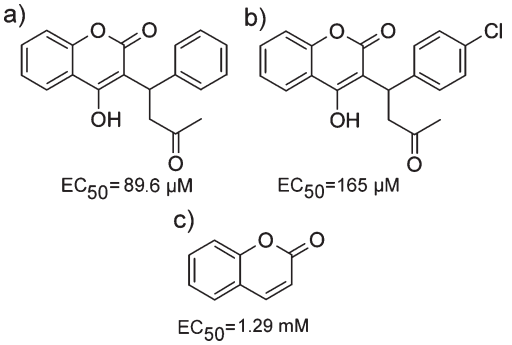

Figure 2. Compounds used in the radioligand displacement studies: (a) warfarin, (b) coumachlor, and (c) coumarin. Values (EC_{50}) presented below each structure are average concentrations required for displacement of 50% of the total amount of radiolabeled warfarin bound to the polymer. All experiments were performed using 0.75 nM [^{3}H]-(*S*)-warfarin.

radiolabeled warfarin was displaced using unlabeled warfarin and the structurally related compounds coumachlor and coumarin (Figure 2). Studies were performed using toluene as a rebinding medium to allow direct examination of the role of the electrostatic interactions between warfarin and polymer functionalities. Interestingly, evaluation of displacement data revealed that the MAA-MIP was able to selectively rebind warfarin. This was supported by a lowest value of the apparent EC_{50} (effective concentration needed for 50% displacement of the amount of radiolabeled warfarin) of 89.6 μM (Figure 3). The somewhat weaker rebinding of the analogue coumachlor associated with an apparent EC_{50} = 165 μM reflected the steric hindrance of the chlorine group, which makes coumachlor probably too large to gain effective access fit in the warfarin imprints. This result, together with others demonstrating the discrimination of ligands differing by no more than a methyl or a methylene group,[33,36] underscores the high degree of ligand fidelity achievable using MIPs. The limited recognition of

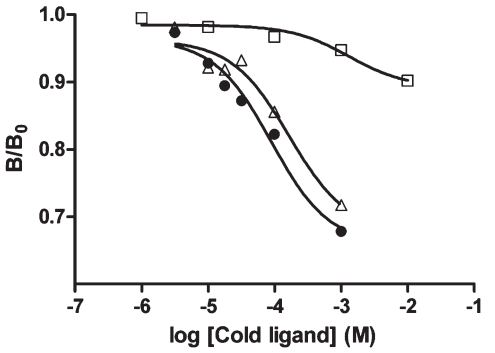

Figure 3. Competitive displacement studies of 0.75 nM [^{3}H]-(*S*)-warfarin binding to 7 mg mL^{-1} of MAA-MIP with increasing concentrations of unlabeled cold ligands: warfarin (●, log EC_{50} = −4.05 ± 0.17, R^2 = 0.98), coumachlor (△, log EC_{50} = −3.78 ± 0.25, R^2 = 0.96), and coumarin (□, log EC_{50} = −2.89 ± 0.32, R^2 = 0.95). B/B_0 is the ratio of the amount of radiolabeled warfarin bound in the presence of competing cold ligand (B) and in the absence of cold ligand (B_0).

coumarin (EC_{50} = 1.29 mM) can be attributed to the lack of functional groups and extent of favorable van der Waals and contacts. The superior performance of the MAA-MIP leads to its use in subsequent studies. Importantly, the rank order of affinity of the ligands for the MAA-MIP reflects that described by Petitpas et al.[22] for HSA, where the importance of in particular hydrophobic interactions and adverse steric constraints is emphasized. The similarity of the relative behavior of the ligands with that of the protein system suggests that this material offers promise for future applications, for example, in sensor development.

Chromatographic [high-performance liquid chromatography (HPLC)] investigations were made of warfarin-MAA-MIP at higher ligand concentrations. Retention data revealed that the MAA-MIP was again selective for the template when binding in chloroform. However, upon switching to the more polar acetonitrile, a significant loss in retention was observed for all ligands on both MAA-MIP and MAA-REF (Table 2). This effect was most profound in the case of the templates and was interpreted as arising from acetonitrile competing more favorably for interaction with warfarin and even for carboxylic acid residues in the warfarin-selective sites.

In a final series of chromatographic studies, the MAA-MIP was characterized using frontal chromatography. Using this methodology, the apparent number of binding sites available was calculated to 35 ± 15 nmol mg^{-1} (K_D = 1.7 ± 0.74 mM) for the MAA-MIP and 64 ± 18 nmol mg^{-1} (K_D = 3.0 ± 0.66 mM) for the MAA-REF. These results further demonstrate the presence of imprints in the MAA-MIP. This result may be compared with HSA, which possesses 15 nmol sites per mg protein. Although these data are collected using different techniques and even in different solvents, the number of sites per gram polymer or biopolymer are comparable. Interestingly, the binding of warfarin to the Sudlow I site of HSA has been reported K_D = 3.0 ± 0.3 μM,[37] which is comparable to data for warfarin binding to polymer in acetonitrile, K_D = 0.6 ± 0.1 μM. Collectively, the binding characteristics of the warfarin imprinted MIP are reminiscent of those seen for HSA-warfarin recognition. Interestingly, the Sudlow I site includes a combination of hydrophobic and hydrophilic functionalities, as is the case of the MAA-MIP. Polymers of this type are capable of mixed-mode binding, as demonstrated in previous studies using synthetic receptors for the local anesthetic bupvacaine.[33]

Table 2. Zonal Chromatographic Analyses at 293 K Using Chloroform or Acetonitrile as Mobile Phases[a]

	k'				N	
	MAA-MIP		MAA-REF			
	chloroform	acetonitrile	chloroform	acetonitrile	chloroform	acetonitrile
coumarin	0.63 ± 0.004	0.21 ± 0.011	0.31 ± 0.003	0.15 ± 0.000	0.92	1.16
coumachlor	3.70 ± 0.003	0.45 ± 0.011	2.00 ± 0.009	0.42 ± 0.010	0.83	0.89
warfarin	4.23 ± 0.044	0.41 ± 0.011	1.91 ± 0.004	0.34 ± 0.000	1.00	1.00

[a] Capacity factors, k', are presented as means ± SDs.

A series of studies were undertaken using steady-state fluorescence spectroscopy to examine the kinetics of the warfarin−polymer interaction (Supporting Information, Figure S11 and Table S4). Rate constants for binding to both MIP and REF were in the 10^{-3} s^{-1} range, and the similarity of the data for the two polymers indicates that observed differences in affinity cannot be ascribed to differences in mass-transfer characteristics. Furthermore, the magnitude of the rate constants is indicative of a rapid establishment of equilibrium, which is attributed to the small particle sizes.

These investigations provide an example of the successful use of MD-based full system prepolymerization mixture simulations as a basis for choice of polymer. Application of the approach has contributed to the development of a polymer selective for warfarin, which we believe may be useful for the development of sensor recognition elements or even yield insights for better understanding blood plasma protein regulated transport and how it can be mediated. The contribution of electrostatic interactions to the recognition of the template has been demonstrated and suggests the use of the polymer in nonpolar media-based solid-phase extraction protocols, where the hydrophobic domains in the MIP binding sites would be expected to contribute more to recognition. We have recently described the complex environment-dependent fluorescence spectroscopic behavior of warfarin[38,39] and its application as a molecular probe.[40] Attempts to combine polymer binding with fluorescence detection are currently underway in our laboratory.

Conclusions

Here, a combination of NMR and MD techniques has been used to develop a MIP with selective recognition for the anticoagulant warfarin. Importantly, the MD studies demonstrated two-point template recognition for the functional monomer MAA but only single point for 4VP. Polymer synthesis confirmed the superiority of the MAA polymer in terms of selectivity as demonstrated by radioligand binding studies, where differentiation of warfarin from the closely related analogue coumachlor was readily achieved. Warfarin−polymer binding has been compared with that at the Sudlow I site of HSA. In both cases, a combination of electrostatic and hydrophobic interactions governs the recognition of the anticoagulant. We propose that these Sudlow I site mimics may provide the basis for models for understanding and steering blood plasma protein-regulated transport processes or even for the development of warfarin sensors.

Experimental Section

Chemicals. Racemic mixtures of warfarin [3-(α-acetonylbenzyl)-4-hydroxycoumarin, ≥98% thin-layer chromatography (TLC)] and coumachlor (3-(α-acetonyl-4-chlorobenzyl)-4-hydroxycoumarin, ≥98% HPLC) were both purchased from Sigma-Aldrich (St. Louis, MO). Coumarin (≥97% UV) was obtained from Fluka (Buchs, Germany). MAA (KeboLab, Täby, Sweden) and 4VP (Sigma-Aldrich) were distilled immediately prior to use. EDMA (≥98% GC) was from Sigma-Aldrich (Steinheim, Germany). AIBN [2,2′-azobis-(2-isobutyronitrile)] was from Janssen Chimica (Geel, Belgium). [^{3}H]-(*S*)-Warfarin (activity 16 Ci $mmol^{-1}$) was purchased from Moravek Biochemicals Inc. (California). Pyridine-d_5 (99 atom % D) acetic-d_3-acid-*d* (99.9 atom % D), acetonitrile-d_3 (99.9 atom % D), and chloroform-*d* (99.9 atom % D, containing 0.03% v/v TMS) were obtained from Aldrich Chemical Co. (Milwaukee, WI). All solvents were of analytical grade and were used as received.

^{1}H NMR Spectroscopy. Isomers of warfarin in chloroform-*d* were resolved using one- or two-dimensional ^{1}H COSY and NOESY experiments (Supporting Information, Figures S1−S3) using 34 mM (700 μL) solutions of warfarin. NMR experiments were performed on a VARIAN spectrometer operating at 500 MHz or an AC-250 Bruker spectrometer operating at 250 MHz at 298 K.

Titrations. To an NMR tube containing 34 mM warfarin dissolved in chloroform-*d*, aliquots of acetic-d_3-acid-*d* (8.73 M) or pyridine-d_5 (2.36 M) containing a constant concentration of warfarin (34 mM) was added, and spectra were acquired after each sequential addition. A corresponding reference series was obtained using solvent in the absence of either acetic-d_3-acid-*d* or pyridine-d_5. For all studies, changes in the chemical shifts, $\Delta\delta$ (ppm), were examined at 298 K. Equilibrium dissociation constants, K_D, were determined using the one site binding model available within the software GraphPad Prism (version 4.00, GraphPad Software, United States). Free induction decay spectra were processed using MestReC for Windows (v. 4.1.1.0).

Continuous Variation. Two stock solutions were prepared in chloroform-*d*, the first with warfarin (34 mM) and the second with acetic-d_3-acid-*d* (34 mM). The two stock solutions were subsequently mixed in terms of warfarin molar fraction ranging from 0 to 1.0. Plotting the different fractions (f_T) versus the absolute changes in chemical shift ($\Delta\delta$) × f_T of the different protons studied, and the stoichiometric relationship between the template and the functional monomer were investigated.

MD Simulations. The extent and nature of functional monomer and warfarin complexation in chloroform during the prepolymerization stage were simulated using the protocol previously described.[28] Mixtures were designed and built according to the isomeric distribution observed by ^{1}H NMR in chloroform; see the Supporting Information (Table S2). In all simulations, nonbonded interactions were treated using a 9.0 Å cutoff. The temperature was maintained at 293 K. The time step was 2 fs, and data were collected for a total production phase of 5 ns. Molecular graphics images were produced using the UCSF Chimera package from the Resource for Biocomputing, Visualization, and Informatics at the University of California, San Francisco (supported by NIH P41 RR-01081).

Polymer Syntheses. In a typical polymer synthesis, a mixture of warfarin (0.695 mmol), MAA (8.35 mmol), and EDMA (38 mmol) was mixed together with AIBN (1.06 mmol, 1.3 mol % of the total polymerizable methacrylate units) in chloroform (1.6 × the volume of the monomers). MIPs were also made using 4VP, instead of MAA, as the functional monomer, using half the amount of template (0.348 mmol); however, the stoichiometries constantly were maintained. Nonimprinted REFs were prepared identically, although in the absence of the warfarin.

Polymerization mixtures were sparged with nitrogen for 20 min on ice and were then sealed and placed under a UV source (365 nm, 281 K, 24 h). The resultant bulk polymers were manually ground and initially dry-sieved through a 63 (MAA) or 50 μm (4VP) sieve. Second, all polymer particles were wet-sieved (acetone) through a 25 μm sieve. Finally, the fine particles were removed by repeated sedimentation from acetone (5 × 200 mL). Polymer particles (~4 g, 25−63 μm for the MAA polymers, and ~2 g, 25−50 μm for the 4VP system) were slurry packed into HPLC columns and subsequently washed as previously described.[33] The polymers were emptied from the column, dried for 24 h at 323 K, and stored at room temperature in a desiccator until further use.

Polymer Characterization. Dry polymers were characterized using BET analyses (Department of Chemical Engineering, Kemicentrum, University of Lund, Sweden) and FT-IR spectroscopy (diffuse reflectance experiments were performed on an Avatar 320 FT-IR spectrophotometer using 50 mg of dry polymer samples in KBr). SEM (Jeol, JSM-840) was performed to examine the surfaces of the imprinted and REF particles. Prior to imaging, particles were coated with a 12 nm layer of platinum using a turbo-sputter coater (BIO-RAD, Polaron Division, E6700) with a magnetron sputtering system. Micrographs were obtained at an accelerating voltage of 10 kV.

Radioligand Binding. Experiments were performed at 293 K in toluene. The tubes were incubated (3 h) on a rocking table and then centrifuged (8000*g*, 5 min). The supernatant (600 μL) was removed from each tube, mixed with scintillation cocktail (Beckman Ready Safe, 2 mL), and counted (2 min, Packard Tri-Carb 2100TR liquid scintillation counter).

Polymer Titrations. The amount of polymer in each sample was in the range from 0.5 to 40 mg. [^{3}H]-(*S*)-Warfarin (0.75 nM) and appropriate amounts of solvent were added to afford a total volume of 1.0 mL.

Competitive Binding. MAA polymer (7.0 mg mL^{-1}) in toluene was incubated together with [^{3}H]-(*S*)-warfarin (0.75 nM) and competing ligands in a concentration range from 1.0 μM to 1.0 mM.

HPLC. MAA polymers were suspended in chloroform−acetonitrile (85:15, v/v, 75 mL) in a slurry reservoir and packed into stainless steel columns (100 mm × 4.6 mm for zonal and 50 mm × 2.1 mm for frontal analyses) at 290 bar using a single action reciprocating plunger pump (Haskel Engineering Supply Co., United States). Acetone was used as the packing solvent. Chromatographic evaluations were performed at 293 K.

Zonal Chromatography. Studies using chloroform as mobile phase were performed using a Hewlett-Packard system comprised of a HP 1050 series pump, VW detector, and an autosampler. Studies using acetonitrile as mobile phase were performed on instrumentation comprised of a Merck Hitachi D-7000 Interface, an L-7455 diode array detector, an L-7200 autosampler, an L-7100 pump, and an L-7612 solvent degasser.

Chromatographic evaluation of the polymers was performed using a flow rate of 1.0 mL min^{-1}. Samples (1.0 mM solutions of warfarin, coumachlor, or coumarin) were injected (triplicate studies of 2 μL volumes). Void volumes were determined using injections (2 μL) of cyclohexane. Capacity factors (k') were determined using retention volumes (V) and the void volume (V_0) using the relationship $k' = (V - V_0)/V_0$. Imprinting factors (IF) were calculated as the ratio between the capacity factor on the MIP and the capacity factor on the REF. By definition, an IF > 1 implies the existence of an imprinting effect. To assess the ligand selectivity of the MIP, the normalized retention factor (N) was determined using the formula:

$$N = \frac{k_{\text{test,imp}}'}{k_{\text{test,ref}}'} \times \frac{k_{\text{imp,ref}}'}{k_{\text{imp,imp}}'} = \frac{\text{IF}_{\text{test}}}{\text{IF}_{\text{imp}}} \quad (1)$$

where the capacity factor of a test substance on either a MIP ($k_{\text{test,imp}}'$) or a REF ($k_{\text{test,ref}}'$) is compared with the same factor observed for the template structure. The MIP is defined to be template selective if $N < 1$ for any analyzed test substance except for the template structure where $N = 1$.

Frontal Chromatography. Experiments were performed on the polymers using chloroform as the mobile phase according to the method described by Kasai et al.[41] (Merck HPLC system as described above). Sample solutions (0.01, 0.05, 0.1, and 0.5 mM warfarin or 5% cyclohexane) were continuously pumped through the column until a stable plateau was achieved. All results are presented as averages from triplicate experiments.

Fluorescence Spectroscopy—Steady-State Studies. Experiments were performed on a Fluoromax-2 spectrofluorometer (ISA Jobin Yvon-SPEX, United States). Fluorescence emission spectra were recorded with an excitation wavelength set to 295 nm, and the emission spectra were corrected for the wavelength dependence of the detection system and Xe lamp (150 W) fluctuations. To reduce the impact of light scattered by the polymer particles, a cutoff filter of 389 nm was placed in front of the emission monochromator slit with a 1 nm spectral bandwidth. The excitation monochromator spectral bandwidth was set to 5 nm. The emission spectrum of warfarin in acetonitrile demonstrated a maximum at 410 nm. The fluorescence spectral form was shown to be independent of excitation wavelength, that is, 295, 305 and 330 nm, which ensured the purity of the sample.

Kinetics Studies. Fluorescence spectroscopy time-based scans were used to estimate the kinetics of the warfarin binding to the MAA polymers in acetonitrile. Fluorescence intensity measurements were started directly after the addition of warfarin (0.5 μM) to the polymer solutions (0.5 mg mL^{-1}). Prior to fluorescence measurements, polymer particles were allowed to swell for at least 20 h in acetonitrile. All measurements were performed under continuous stirring using a standard quartz cuvette (1 cm path length, total volume 2 mL at room temperature). Upon recording spectra, the increment step was 1 nm, and the integration time was 0.5 s. In these experiments, the sample fluorescence signal was measured with excitation at 295 nm and emission detection at 440 nm. The time increment employed was 1 min, with an illumination time of 5 s, collecting data for a total time of 150 min. To avoid photo bleaching, the shutter was closed between each excitation period.

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Supporting Information Available: Experimental procedures together with additional figures and tables presenting experimental results. This material is available free of charge via the Internet at http://pubs.acs.org.

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