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JOURNAL OF THE AMERICAN CHEMICAL SOCIETY
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A Connection between the Binding Properties of Imprinted and Nonimprinted Polymers: A Change of Perspective in Molecular Imprinting

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Supporting Information

ABSTRACT: In the current paradigm for molecular imprinting, the imprinted binding sites exist as a consequence of the polymerization process around templates, and the properties of nonimprinted polymers (NIPs) have largely been overlooked. Thus, nothing can be affirmed a priori concerning the binding properties of NIPs. We propose an alternative view where the imprinting effect is due to the presence of a template molecule that enhances the pre-existing binding properties of a polymer. If a NIP shows no binding properties toward a target molecule, the corresponding imprinted polymer (MIP) will show a weak imprinting effect. On the other hand, if a NIP shows binding properties toward a target molecule, the corresponding MIP will show a significant imprinting effect. To verify this hypothesis, we prepared a 96-member combinatorial polymeric library in the absence of any template molecule. This library was screened for several potential ligands, and with no exceptions, the composition of the best-binding NIP produced a MIP with excellent binding properties, whereas a low-binding NIP formulation produced a MIP with comparable low binding. To validate these results, the binding properties toward naproxen and ibuprofen were measured for two combinatorial libraries of polymers prepared in the presence (MIP library) and the absence (NIP library) of the template molecule. The experiment's results showed a correlation between the apparent affinity constants measured for the NIP and MIP libraries, confirming the proposed hypothesis. Moreover, for closely related molecules, it was shown that binding selectivity is an emergent property derived from the imprinting process and not a property of NIPs.

INTRODUCTION

Molecularly imprinted polymers (MIPs) can be obtained by the polymerization of a mixture of cross-linkers and functional monomers in the presence of a template dissolved in a proper porogenic solvent.[1] The nature of the resulting material and its binding properties are influenced not only by the composition of the prepolymerization mixture[2] but also by the experimental conditions employed, such as the type and amount of radical initiator used, the polymerization temperature, the type of polymerization mechanism, and so forth.[3] It is often assumed that the template molecule plays a pivotal role and that cross-linkers, functional monomers, and porogenic solvents should be chosen by taking into account the chemical properties of the template. Thus, the current paradigm describing the origins of the molecular imprinting mechanism can be illustrated by the well-known empirical model where the imprinted binding site exists as a direct consequence of the polymerization of several monomers around the template molecule. This description seems to be confirmed not only by the huge large amount of papers reported in the last 20 years but also by successful in silico simulations of several imprinted systems.[4] Moreover, the existence of imprinted sites is supported by a large amount of experimental data indicating how they act as reversible binding sites with well-defined (and surprisingly complex) thermodynamic and kinetic behaviors influenced by steric and electronic features of the template molecule.[5]

In the current paradigm, there has not been much attention paid to the properties of nonimprinted polymers (NIPs). In fact, any imprinting effect in a polymer is the consequence of the presence of the template molecule in the polymerization mixture and its interactions with the mixture components. Thus, it is very difficult to make reliable predictions about the binding properties of NIPs prepared without any template molecule. However, this paradigm seems to be challenged in some manner by papers describing MIPs or NIPs with unexpected molecular recognition properties.[6] Moreover, several papers have recently been published about polymers that are characterized by good selectivity and binding properties toward small organic molecular targets[7] or even larger peptides[8] prepared without the use of a template.

On the basis of these facts, we think that an alternative view of molecular imprinting is possible. In this hypothesis, illustrated in Figure 1, the presence of the template molecule in the prepolymerization mixture acts to enhance binding properties that already preexist in a NIP. As a consequence, if a NIP shows

Received: June 17, 2011
Published: December 21, 2011

   dx.doi.org/10.1021/ja205632t | J. Am. Chem.Soc. 2012, 134, 1513–1518

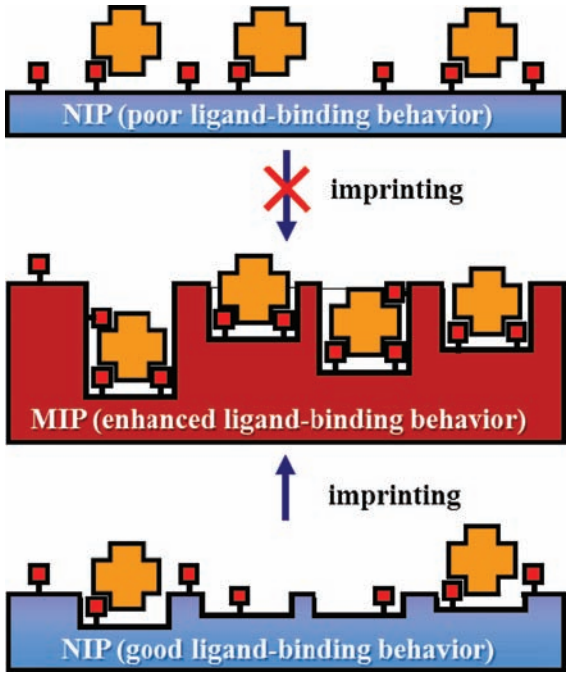

Figure 1. The working hypothesis. The presence of the template molecule in the prepolymerization mixture acts to enhance binding properties that already exist in a NIP. Thus, if a NIP shows limited binding properties toward a target molecule, the corresponding MIP will show a weak imprinting effect, if any. On the contrary, if the NIP shows marked binding properties toward a target molecule, the corresponding MIP will show a significant imprinting effect.

no binding properties toward a target molecule, the corresponding MIP will show a weak imprinting effect, if any. On the other hand, if the NIP shows binding properties toward a target molecule, the corresponding MIP will show a significant imprinting effect.

To verify this hypothesis, in this work we prepared a 96-member combinatorial polymeric library in the absence of any template molecule (the NIP library). This library was screened for several potential ligands, and with no exception, the composition of the best-binding NIP produced a MIP with excellent binding properties, whereas a low-binding NIP formulation produced a MIP with comparable low binding. To validate these results, the equilibrium binding properties (affinity constant, binding site density) toward naproxen were measured for two combinatorial libraries of polymers, prepared in the presence (MIP library) and the absence (NIP library) of the template molecule by varying the functional monomer, the cross-linker, and the porogen. The screening of 96 different polymers confirmed a clear positive correlation between the binding properties measured for the NIP and MIP libraries.

RESULTS AND DISCUSSION

Synthesis and Screening of the Polymeric Combinatorial Library. Our hypothesis of a relationship between the binding properties of imprinted and nonimprinted polymers was verified by preparing a nonimprinted library of 96 elements and screening it for the binding of several ligands. After that, the best-binding nonimprinted polymers were compared with the related imprinted polymers.

To ensure a large degree of molecular diversity in the composition of the polymers, we combined very different functional monomers, cross-linkers, and porogenic solvents, all previously reported in the literature as components of successful MIPs.[9] Neutral (acrylamide, 2-hydroxyethylmethacrylate), acidic (methacrylic acid), and basic (4-vinylpyridine) compounds were used as functional monomers, while cross-linkers were selected in terms of the number of possible polymerizable groups: two (divinylbenzene, ethylene dimethacrylate, glycerol dimethacrylate), three (pentaerithrytole triacrylate, trimethylolpropane trimethacrylate), and four (pentaerithrytole tetraacrylate). Porogenic solvents were selected to represent different typologies of organic solvents, including those with aromatic (toluene), hydrophobic (chloroform), and hydrophilic (acetonitrile, tetrahydrofuran) character.

In an attempt to ensure that any relationship between the molecular recognition properties of the imprinted and nonimprinted polymers was not the spurious effect of chance, the NIP library was screened in such a way as to be sure that the degree of molecular diversity in the ligand structures was sufficiently wide. Chloramphenicol and cortisol are neutral molecules, while diclofenac, ibuprofen, and naproxen are acids with pK values of 4−5, bisphenol A and theophylline are very weak acids, and metribuzin and pyrimethanil are weak bases. The hydrophobicity covers a very large interval of logP values, ranging from −0.02 for theophylline, an essentially hydrophilic molecule, to 4.51 for diclofenac, which in fully protonated form is very hydrophobic. Moreover, all of the considered ligands have been previously reported in the literature as template molecules; the corresponding imprinted polymers have been extensively studied, and the binding behavior is very well-known.[10]

The effect of the large molecular diversity represented by the panel of ligands is well illustrated in Figure 2, where the box

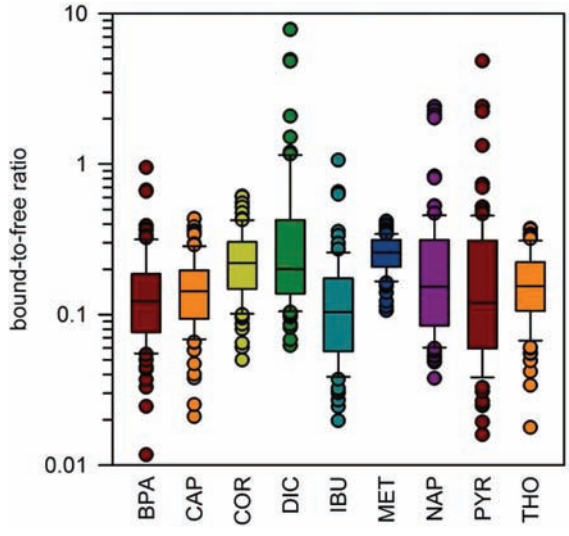

Figure 2. Bound-to-free ratio (B/F) values measured for each of the ligands by overnight incubation at 4 °C of 10 mg of polymer suspended in 200 μL of 50 μg/mL ligand solution in acetonitrile. See note S1 in the Supporting Information (SI) for the statistical meaning of this plot. Definitions of the acronyms are given in Chart S1 in the SI.

plot reports the spreading of the bound-to-free ratio (B/F) values measured for each of the ligands. It is possible to see that different ligands bind in very different ways, with B/F values between 0.05 and 0.5 (first to third percentile), ranging from results dispersed at wide intervals of B/F values (diclofenac and pyrimethanil) to results present at relatively narrow intervals of B/F values (chloramphenicol, cortisol, metribuzin, and theo-

dx.doi.org/10.1021/ja205632t | J. Am. Chem.Soc. 2012, 134, 1513−1518

Table 1. Bound-to-Free Ratio (B/F) Values Measured for Selected Polymers Presenting the Best and the Worst Ligand Binding in Accordance with the Binding Screening of the NIP Library

	best-binding polymer			worst-binding polymer		
ligand	polymer formulation[a]	MIP B/F	NIP B/F	polymer formulation[a]	MIP B/F	NIP B/F
bisphenol A	4VP–DVB–$CHCl_3$	1.49	0.95	HEMA–DVB–MeCN	0.02	0.02
chloramphenicol	4VP–ETA– $CHCl_3$	0.63	0.43	MAA–PETA–TOL	0.02	0.02
cortisol	4VP–DVB–$CHCl_3$	1.37	0.55	HEMA–PETA–TOL	0.07	0.05
diclofenac	4VP–DVB–MeCN	32.1	7.84	MAA–PETA–TOL	0.07	0.06
ibuprofen	4VP–DVB–THF	1.69	1.06	AM–GDMA–TOL	0.03	0.02
metribuzin	4VP–GDMA–THF	0.61	0.42	MAA–DVB–MeCN	0.13	0.11
naproxen	4VP–DVB–TOL	1.69	0.91	HEMA–EDMA–TOL	0.08	0.05
pyrimethanil	MAA–DVB–THF	22.8	4.84	HEMA–GDMA–TOL	0.02	0.01
theophylline	AM–DVB–$CHCl_3$	0.53	0.37	MAA–TRIM–MeCN	0.04	0.02

[a]Definitions of the acronyms are given in Table S1 in the SI.

phylline), with some intermediate situations (bisphenol A, ibuprofen, naproxen, and pyrimethanil).

Comparison of the Binding Properties of Imprinted and Nonimprinted Polymers. The B/F results related to the different ligands were examined to identify the composition of the best- and worst-binding polymers for each of the ligands considered, and the ligand binding was measured for the corresponding imprinted polymers. The B/F ratio for each of the polymer pairs is reported in Table 1. Despite the difficulty of exactly comparing binding data for extreme B/F values (B/F < 0.1 or B/F > 10), from these results it is nevertheless possible to observe that without exception, the composition of the best-binding NIP produced a MIP with excellent binding properties, as characterized by a marked increase of the ligand binding (evaluated as the increase in the difference between the B/F values measured for the NIP and MIP), whereas a low-binding NIP formulation produced a MIP with comparably low ligand binding. Interestingly, it seems that the pair 4-vinylpyridine/divinylbenzene represent the optimal functional monomer/cross-linker combination, as it is present in five of the nine formulations corresponding to high-binding polymers (polymers binding bisphenol A, cortisol, diclofenac, ibuprofen, and naproxen), and that 4-vinylpyridine (but not divinylbenznene) is present in two other formulations (polymers binding chloramphenicol and metribuzin). This result can be related to the fact that six out of the nine tested templates are molecules with carboxyl or hydroxyl substituents, which are known to interact with the pyridine ring through hydrogen-bonding or ion-pair interactions,[10] and that metribuzin, a weakly acidic molecule, is both a good hydrogen-bond acceptor and donor and thus is able to interact with 4-vinylpyridine, a strong hydrogen-bond acceptor. On the other hand, it seems impossible to identify clearly a functional monomer/cross-linker combination typical of formulations giving poorly binding polymers.

Comparison of the Binding Isotherms of Imprinted and Nonimprinted Polymers. The measurement of the B/F ratios for MIPs and NIPs reported in the previous section is related to a single point in a binding isotherm, measured for a ligand concentration of 50 μg/mL. Thus, only indirect information on the binding properties of the polymers can be obtained. To provide a better validation of these results, it was decided to gather direct information on the ligand binding properties (i.e., apparent affinity constants K_{eq} and binding site densities B_{max}) by measuring the whole binding isotherm for two combinatorial libraries of polymers prepared in the presence (MIP library) and the absence (NIP library) of naproxen. Despite the well-known complexity of the binding behavior of MIPs,[5f] a simple Langmuir model was chosen to limit the number of the experimental points necessary to obtain accurate estimates of equation parameters.

The comparison of the K_{eq} values measured on the MIP and NIP libraries by applying a Mann–Whitney rank sum test (Figure 3) shows that the numerical difference between the two

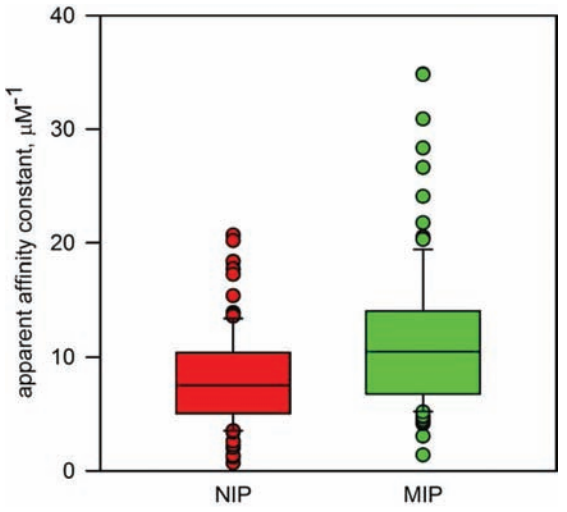

Figure 3. Comparison of apparent affinity constants (K_{eq}) measured for naproxen on the MIP and NIP libraries based on a Mann–Whitney rank sum test. See note S1 in the SI for the statistical meaning of this plot.

groups is greater than would be expected by chance (P < 0.000001), thus confirming that there is a statistically significant difference between the distributions of K_{eq} values in the MIP and NIP libraries. From the plot reported in Figure 4, it is possible to observe a statistically significant direct relationship between the K_{eq} values for MIPs and NIPs, as expressed by the following linear regression model of K_{eq}(MIP) versus K_{eq}(NIP):

$$K_{eq}(\mathrm{MIP})_{\mathrm{naproxen}} = (0.298 \pm 0.753) + (1.39 \pm 0.0832)K_{eq}(\mathrm{NIP})_{\mathrm{naproxen}}$$

$$(n = 96,\ r^2 = 0.748,\ s = 3.29,\ F = 278.4,\ P < 0.0001) \quad (1)$$

It should be noted that the slope of the regression line is greater than unity, indicating not only that there is a marked difference between the K_{eq} values measured for the NIP and MIP libraries but also that the K_{eq} values measured for the MIP library

dx.doi.org/10.1021/ja205632t | J. Am. Chem.Soc. 2012, 134, 1513–1518

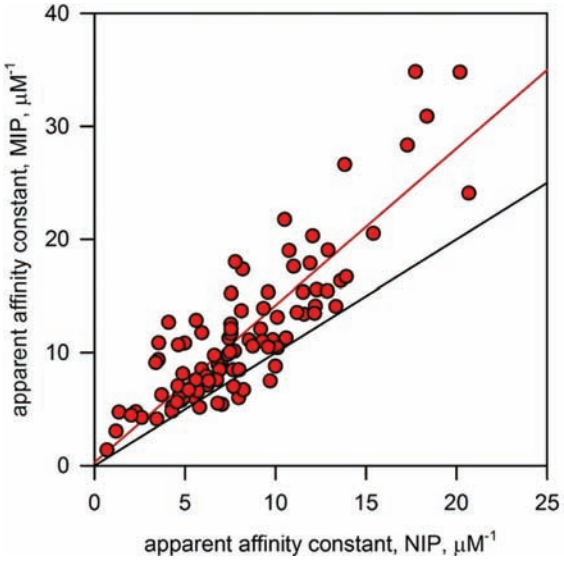

Figure 4. Relationship between the apparent affinity constants (K_{eq}) measured for naproxen on the MIP and NIP libraries. The red line indicates the linear regression model of K_{eq}(MIP) vs K_{eq}(NIP). The black line represents the upper edge of the K_{eq}(MIP) < K_{eq}(NIP) region.

increase proportionally with the increase in the K_{eq} values for the NIP library.

As regards B_{max}, the comparison of the values measured on the MIP and NIP libraries (Figure 5) using the same test as

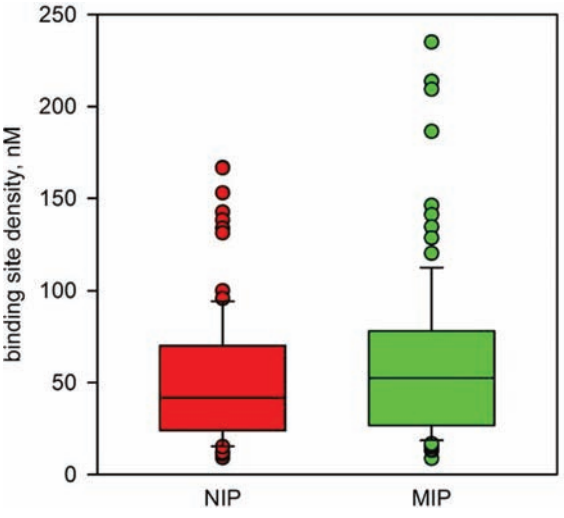

Figure 5. Comparison of the binding site density (B_{max}) values measured for naproxen on the MIP and NIP libraries based on a Mann–Whitney rank sum test.

for K_{eq} shows that the numerical difference between the two groups is not greater than would be expected by chance (P = 0.1426), confirming that there is not a significant difference between the B_{max} values measured for the NIP and MIP libraries. Thus, it seems that the main difference between MIPs and NIPs is related to differences in the magnitude of the binding affinity rather than to differences in the number of available binding sites.

As in the case of the K_{eq}(MIP) versus K_{eq}(NIP) model, a statistically significant linear regression of B_{max}(MIP) versus

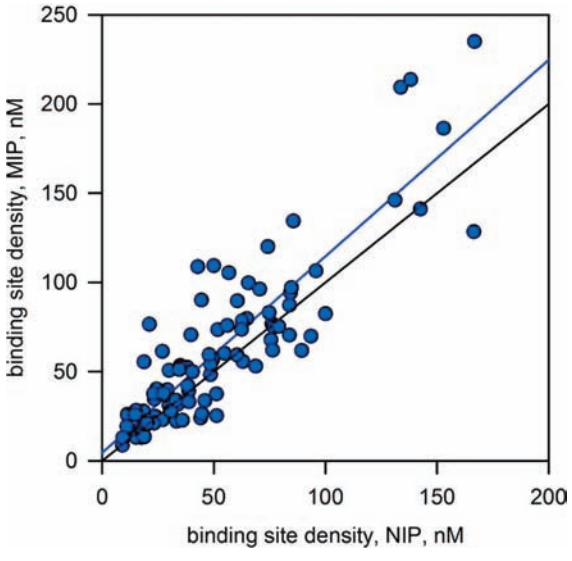

Figure 6. Relationship between the binding site density (B_{max}) values for naproxen measured on the MIP and NIP libraries. The blue line indicates the linear regression model of B_{max}(MIP) vs B_{max}(NIP). The black line represents the upper edge of the B_{max}(MIP) < B_{max}(NIP) region.

B_{max}(NIP), whose plot is shown in Figure 6, is described in the following equation:

$$B_{max}(\text{MIP})_{\text{naproxen}} = (4.53 \pm 3.90) + (1.10 \pm 0.0624) B_{max}(\text{NIP})_{\text{naproxen}}$$

$$(n = 96,\ r^2 = 0.768,\ s = 21.9,\ F = 311.6,\ P < 0.0001) \quad (2)$$

Considering that plots reported in Figures 4 and 6 show the presence of linear models correlating the binding properties of NIPs and MIPs, it is clear that there are many low-K_{eq}, low-B_{max} MIPs corresponding to low-K_{eq}, low-B_{max} NIPs and a more limited number of high-K_{eq}, high-B_{max} MIPs corresponding to high-K_{eq}, high-B_{max} NIPs but that there are no high-K_{eq}, high-B_{max} MIPs with compositions corresponding to low-K_{eq}, low-B_{max} NIPs. This confirms our working hypothesis: *If a NIP shows limited binding properties toward a target molecule, the corresponding MIP will show a weak imprinting effect, if any. On the contrary, if the NIP shows marked binding properties toward a target molecule, the corresponding MIP will show a significant imprinting effect.*

Binding Selectivity of Imprinted and Nonimprinted Polymers. Naproxen was chosen as an imprint molecule because it was possible to compare its binding properties to ibuprofen, a closely related ligand already examined in the preliminary screening of the NIP library. Thus, the binding selectivity was studied by comparing the measured values of K_{eq} and B_{max} for naproxen and ibuprofen on NIP and (naproxen-imprinted) MIP libraries.

The statistical comparison of K_{eq} values measured for ibuprofen on the NIP and (naproxen-imprinted) MIP libraries (Figure 7) shows that the numerical difference between the two groups of data is greater than would be expected by chance (P = 0.016), thus confirming that also for ibuprofen there is a statistically significant difference between the distribution of K_{eq} values in the NIP and (naproxen-imprinted) MIP libraries and that this difference can be attributed to the recognition of the ibuprofen molecules by the imprinted library. On the contrary, the comparison of the B_{max} values measured for ibuprofen on

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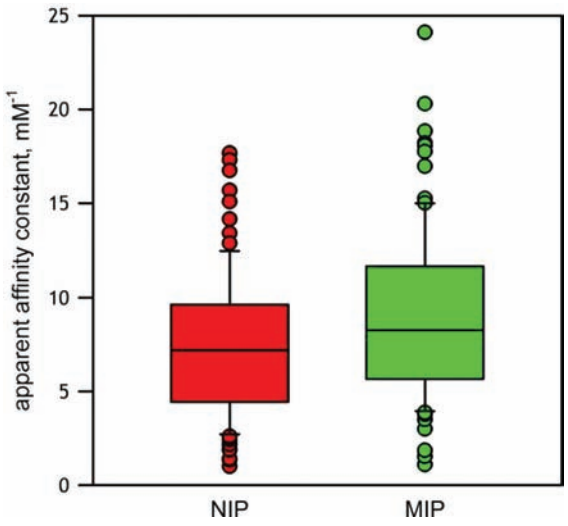

Figure 7. Comparison of apparent affinity constant (K_{eq}) values measured for ibuprofen on the NIP and (naproxen-imprinted) MIP libraries based on a Mann−Whitney rank sum test.

the NIP and (naproxen-imprinted) MIP libraries using the same test as for the K_{eq} values (Figure 8) shows that the

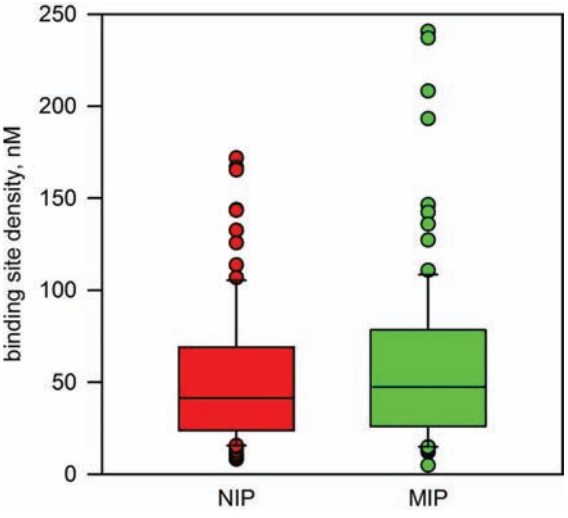

Figure 8. Comparison of the binding site density (B_{max}) values measured for ibuprofen on the NIP and (naproxen-imprinted) MIP libraries based on a Mann−Whitney rank sum test.

numerical difference between the two groups is not greater than would be expected by chance (P = 0.284), confirming what was observed for naproxen: there is not a significant difference between the B_{max} values measured for the NIP and MIP libraries.

As the polymer selectivity seems to be controlled by the ligand affinity only, while the number of binding sites seems to be unimportant, it is interesting to make a direct comparison of the corresponding linear regression models of K_{eq}(ibuprofen) versus K_{eq}(naproxen) calculated for NIP and MIP libraries:

$$K_{eq}(\text{NIP})_{\text{naproxen}} = (1.24 \pm 0.481) + (0.926 \pm 0.058)K_{eq}(\text{NIP})_{\text{ibuprofen}}$$

$$(n = 96,\ r^2 = 0.731,\ s = 2.12,\ F = 255.4,\ P < 0.0001) \quad (3)$$

$$K_{eq}(\text{MIP})_{\text{naproxen}} = (0.0114 \pm 0.562) + (1.28 \pm 0.0735)K_{eq}(\text{MIP})_{\text{ibuprofen}}$$

$$(n = 96,\ r^2 = 0.764,\ s = 3.19,\ F = 303.9,\ P < 0.0001) \quad (4)$$

From the plot reported in Figure 9, it is possible to observe that the numerical value for the slope of the regression model

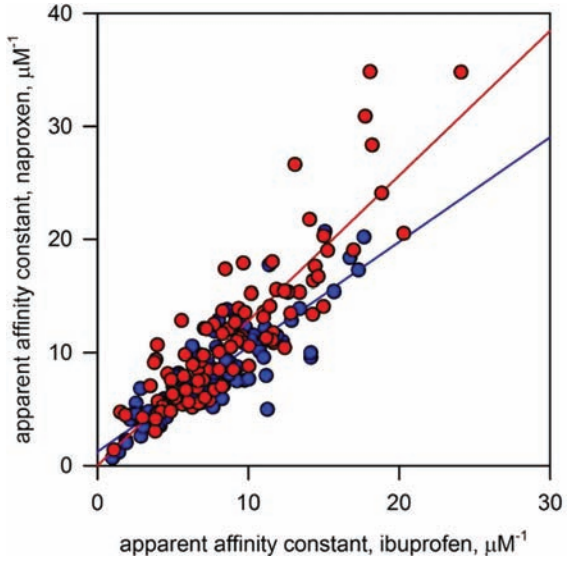

Figure 9. Relationships between the apparent affinity constant (K_{eq}) values measured for ibuprofen and naproxen on the MIP (red ●) and NIP (blue ●) libraries. The solid lines indicate the linear regression models for K_{eq}(ibuprofen) vs K_{eq}(naproxen) calculated for the NIP (blue) and MIP (red) libraries.

calculated for the NIP library (eq 3) is ∼1, indicating that naproxen and ibuprofen show the same binding behavior and are recognized in the same manner by the NIP library. On the contrary, the regression model calculated for the (naproxen-imprinted) MIP library (eq 4) shows a slope significantly greater than 1, indicating that naproxen is better recognized than ibuprofen. Thus, by analogy to what is known about the capabilities of racemic resolution typical of MIPs imprinted against optically active molecules, it can be assumed that for closely related molecules, the binding selectivity seems to be a molecular recognition property arising from the imprinting process.

■ CONCLUSIONS

The libraries considered in this work can be considered representative of widely used experimental conditions involving small molecules as templates and noncovalent bulk imprinting conditions. Thus, as the current study is not concerned with other different imprinting approaches (e.g., covalent imprinting, ion imprinting, use of large templates such as proteins, etc.), we think that our results can be considered valid and of general value for the noncovalent imprinting approach. The clear and positive correlation between the apparent affinity constants measured both for the NIP and MIP libraries indicates that these libraries share the same binding behavior, confirming our initial hypothesis: in the imprinting process, the presence of the template molecule in the prepolymerization mixture acts to enhance the resulting MIP binding properties that exist in the corresponding NIP.

dx.doi.org/10.1021/ja205632t | J. Am. Chem.Soc. 2012, 134, 1513−1518

As regards the selectivity of the molecular recognition properties, considering strictly related ligands, as in the case for the pair naproxen and ibuprofen, the experimental results reported here confirm what is common knowledge for the imprinting process: selectivity between enantiomeric pairs or structurally related molecules is an emergent property derived from the imprinting process, and NIPs tend to be poorly selective.

Apart from the contribution to a better understanding of the fundamentals of molecular imprinting, we think that these results have some important practical implications in MIP technology. In fact, as NIP and MIP libraries show the same binding behavior, it should be possible with a reasonable rate of success (thus not excluding the possibility that some false positives and false negatives may happen) to identify efficient prepolymerization mixtures to prepare high-binding imprinted polymers simply by screening a NIP library, thus making easier the cumbersome process of optimizing a MIP formulation. In fact, not only is the synthesis of a NIP library much cheaper and simpler than a MIP library, as no template must be used to imprint the polymers and subsequently be extracted, but the same library can be recycled many times to screen for different target ligands, simultaneously or in sequence, without the need to prepare many different MIP libraries. Moreover, the relatively simple accessibility to very large libraries of hundreds of different polymers paves the way to fast screening for exotic polymer formulations involving functional monomers and cross-linkers that are much more different than the "classical" methacrylic acid, 4-vinylpyridine, or ethylene dimethacrylate.

■ ASSOCIATED CONTENT

Supporting Information

Materials and methods, template structures, and composition of the polymeric combinatorial libraries. This material is available free of charge via the Internet at http://pubs.acs.org.

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■ ACKNOWLEDGMENTS

C.B. thanks Prof. Gunther Wulff for his very helpful advice.

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