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A connection between the binding properties of imprinted and non-imprinted polymers: a change of perspective in molecular imprinting

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KEYWORDS. Molecular imprinting, imprinted polymer, non imprinted polymer, molecular recognition, binding isotherm, affinity constant, binding site density, selectivity

Supporting Information Placeholder

ABSTRACT: In the current paradigm about molecular imprinting, the imprinted binding sites exist as a consequence of the polymerization process around templates, and the properties of non-imprinted polymers (NIPs) has been largely overlooked. Thus, nothing can be affirmed a priori on the binding properties of NIPs. We propose an alternative view where the imprinting effect is due to the presence of a template molecule which enhances the pre-existing binding properties of a polymer. If a NIP shows no binding properties towards a target molecule the corresponding imprinted polymer (MIP) will show a weak imprinting effect. On the other hand, if a NIP shows binding properties towards 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 towards 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 constant measured for the NIP- and the MIP-library, 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 pre-polymerization mixture,[2] but also by the experimental conditions employed, such as including the type and the 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 which describes the origins of 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 twenty years, but also by successful in silico simulation of several imprinted systems.[4] Moreover, the existence of imprinted sites is supported by a large amount of experimental data, that indicates 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 non-imprinted polymers (NIP). 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 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 which are characterized by good selectivity and binding properties towards 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 on molecular imprinting is possible. In this hypothesis, illustrated in figure 1, the presence of the template molecule in the

pre-polymerization mixture acts to enhance binding properties that preexist anyway in a NIP. As a consequence, if a NIP shows no binding properties towards a target molecule, the corresponding MIP will show a weak imprinting effect, if any. On the other hand, if the NIP shows binding properties towards 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 (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) towards 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 the MIP-library.

RESULTS AND DISCUSSION

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

To assure 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 literature as components of successful molecularly imprinted polymers.[9] Neutral (acrylamide, 2-hydroxyethylmethacrylate), acid (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 in a way so as to represent different typologies of organic solvent: with aromatic (toluene), hydrophobic (chloroform) and hydrophilic (acetonitrile and tetrahydrofurane) character.

In an attempt to assure that any relationship between the molecular recognition properties of imprinted and non-imprinted polymers was not the spurious effect of chance, the non-imprinted library was screened in such a way 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 of about 4-5, bisphenol A and theophylline very weak acids, and metribuzin and pyrimethanil weak basis. 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, that in fully protonated form is very hydrophobic. Moreover, all the considered ligands have been previously reported in literature as template molecules, and the corresponding imprinted polymers have been largely 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 plot reports the spreading of the bound-to-free (B/F) ratio 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 (1^{st}-3^{rd} 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 theophylline), with some intermediate situations (bisphenol A, ibuprofen, naproxen and pyrimethanil).

Comparison of the binding properties of imprinted and non-imprinted 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 has been measured for the corresponding imprinted polymers. In table 1 the B/F ratio for each of the polymer pairs are reported. Despite the difficulty to exactly compare binding data when the B/F ratio assumes extreme values (B/F<0.1 or B/F>10), from these results it is nevertheless possible to observe that, with no exception, the composition of the best binding NIP produced a MIP with excellent binding properties, characterized by a marked increase of the ligand binding (evalued as the increase of the difference between B/F measured for NIP and MIP), whereas a low binding NIP formulation produced a MIP with comparable 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 5 of 9 formulations corresponding to high binding polymers (polymers binding bisphenol A, cortisol, diclofenac, ibuprofen and naproxen), and that 4-vinylpyridine – but no divinylbenznene - is present in other 2 formulations (polymers binding chloramphenicol and metribuzin). This result can be related to the fact that 6 out of 9 tested templates are molecules with carboxyl or hydroxyl substituents, known to interact with the pyridine ring through hydrogen bond or ion pair interactions,[10] and that metribuzin, a weak acidic molecule, is both a good hydrogen bond acceptor and donor, able to interact with 4-vinylpyridine – a strong hydrogen bond acceptor. On the other hand, it seems impossible to clearly identify a functional monomer / cross-linker combination typical of formulations giving poorly binding polymers.

Comparison of the binding isotherms of imprinted and non-imprinted polymers. The measurement of the B/F ratio 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 better validate these results, it was decided to gather direct information on the ligand binding properties (i.e. apparent affin-

ity constant, K_{eq}, and binding site density, 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,[11] 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 K_{eq} values measured on the MIP- and NIP-libraries applying a Mann-Whitney rank sum test (figure 3) shows that the numerical difference between the two groups is greater than would be expected by chance (P<0.000001), thus confirming that there is a statistically significant difference between the distribution 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 of MIPs and NIPs, expressed by a linear regression model of K_{eq}(MIP) *vs.* K_{eq}(NIP):

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

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

It should be noted that the slope of the regression line is greater than the unit, indicating that not only there is a marked difference between K_{eq} values measured for the NIP- and MIP-library, but that K_{eq} values measured for the MIP-library increases proportionally with the increase of 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) with the same test used 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 B_{max} measured for the NIP- and MIP-library. Thus, it seems that the main difference between MIPs and NIPs will be related to differences in the magnitude of binding affinity, and not to the number of available binding sites.

As in the case for K_{eq}(MIP) *vs.* K_{eq}(NIP) model, a statistically significant linear regression of B_{max}(MIP) *vs.* B_{max}(NIP), whose plot is reported in figure 6, is described in the following equation:

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

$$r^2=0.768,\ n=96,\ s=21.9,\ F=311.6,\ P<0.0001. \qquad (3)$$

Considering that plots reported in figure 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 there are no high-K_{eq}, high-B_{max} MIPs with compositions corresponding to low-K_{eq}, low-B_{max}. This confirms our working hypothesis, *i.e. that if a NIP shows limited binding properties towards a target molecule, the corresponding MIP will show a weak imprinting effect, if any. On the contrary, if the NIP shows marked binding properties towards a target molecule, the corresponding MIP will show a significant imprinting effect.*

Binding selectivity of imprinted and non-imprinted polymers. Naproxen was chosen as an imprint molecule as 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, binding selectivity was studied by comparing the measured values of K_{eq}, and B_{max} of naproxen and ibuprofen on NIP- and (naproxen-imprinted)MIP-libraries.

The statistical comparison of K_{eq} values measured for ibuprofen on the (naproxen-imprinted)MIP- and NIP-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 (naproxen-imprinted)MIP- and NIP-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 the (naproxen-imprinted)MIP- and NIP-libraries with the same test used for K_{eq} values (figure 8) shows that the 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 B_{max} values measured for the NIP- and MIP-library.

As 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) *vs.* K_{eq}(naproxen) calculated for NIP- and MIP-libraries:

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

$$r^2=0.731,\ n=96,\ s=2.12,\ F=255.4,\ P<0.0001 \qquad (4)$$

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

$$r^2=0.764,\ n=96,\ s=3.19,\ F=303.9,\ P<0.0001 \qquad (5)$$

From the plot reported in figure 9, it is possible to observe that the numerical value for the slope of the regression model calculated for the NIP-library (eq.4) is about one, 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.5) shows a slope significantly greater than one, 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 non-covalent bulk imprinting conditions. Thus, as the current study is not concerned with other different imprinting approaches (like covalent imprinting, ion imprinting, use of large templates like proteins and so on), we think that our results can be considered valid and of general value for the non-covalent imprinting approach. The clear and positive correlation between the apparent affinity constants measured both for the NIP- and the MIP-libraries means that these libraries share the same binding behavior, confirming our initial hypothesis; in the imprinting process the presence of the template molecule in the pre-polymerization mixture acts to enhance the resulting MIP binding properties that exist in the corresponding NIP.

As regard the selectivity of the molecular recognition properties, considering strictly related ligands – as in the case for the pair naproxen / ibuprofen - the experimental results reported here confirm what common knowledge of the imprinting process is: 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 that some false positives and false negatives happens) 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 has to be used to imprint the polymers and subsequently 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-linker which are much more different than the "classical" methacrylic acid, 4-vinylpyridine or ethylene dimethacrylate.

TABLES.

Table 1: bound to free ratio measured for selected polymers presenting the best and the worst ligand binding in according with the binding screening of the non imprinted polymeric library

	Best binding polymer			Worst binding polymer		
Ligand	Polymer formulation	B/F MIP	B/F NIP	Polymer formulation	B/F MIP	B/F NIP
Bisphenol A	4VP-DVB-$CHCl_3$	1.49	0.95	HEMA-DVB-MeCN	0.02	0.02
Chloramphenicol	4-VP-PETA- $CHCl_3$	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

FIGURES.

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

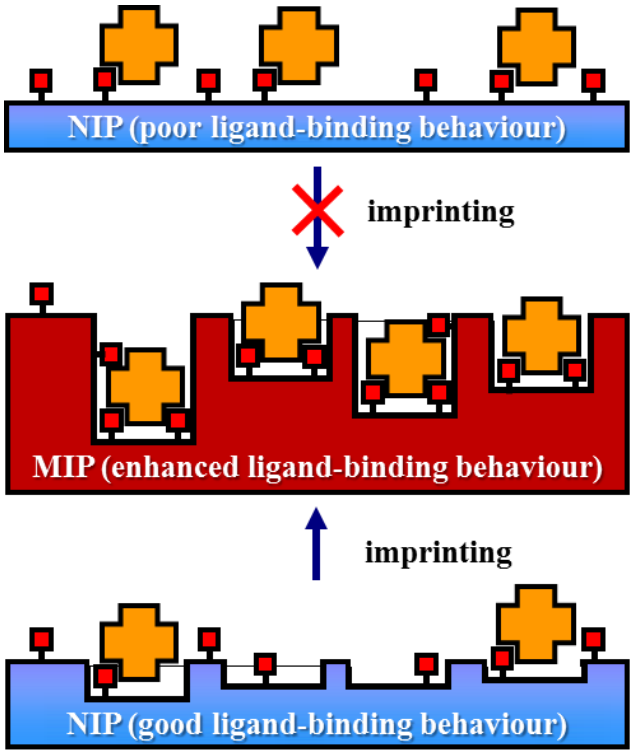


Figure 2. Bound-to-free ratio (B/F) values measured for each of the ligands by overnight incubation at 4 °C of 10 mg of polymer suspended in 200 µl of 50 µg/ml ligand solution in acetonitrile. See supporting informations (note S1) for the statistical meaning of this plot.

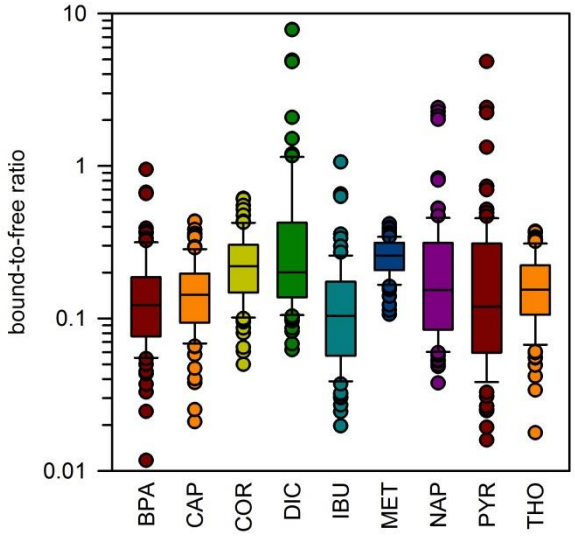


Figure 3. Comparison of apparent affinity constant (K_{eq}) values measured for naproxen on the MIP- and NIP-libraries applying a Mann-Whitney rank sum test. See supporting informations (note S1) for the statistical meaning of this plot.

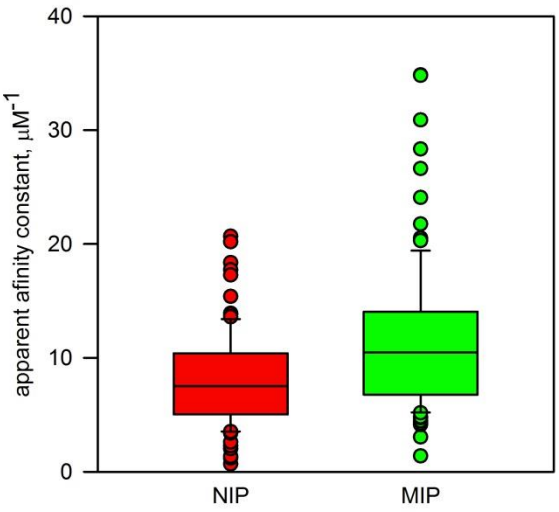


Figure 4. Relationship between the apparent affinity constant (K_{eq}) values measured for naproxen on the MIP- and NIP-libraries. The red line indicates the linear regression model of K_{eq}(MIP) *vs.* K_{eq}(NIP). The black line represents the upper edge for the K_{eq}(MIP) < K_{eq}(NIP) region.

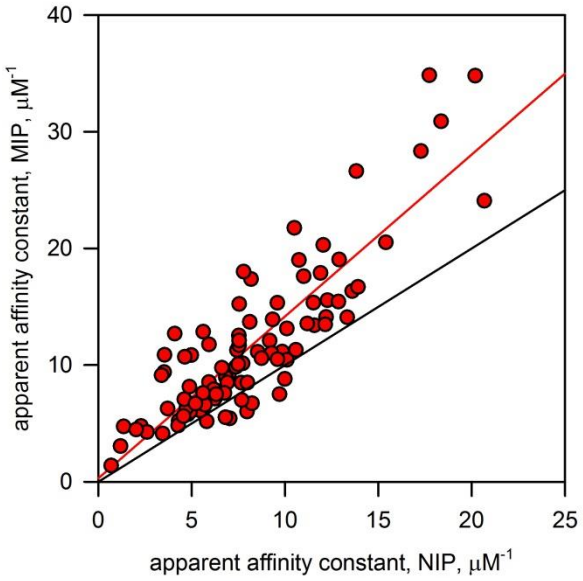


Figure 5. Comparison of the binding site density (B_{max}) values measured for naproxen on the MIP- and NIP-libraries applying a Mann-Whitney rank sum test.

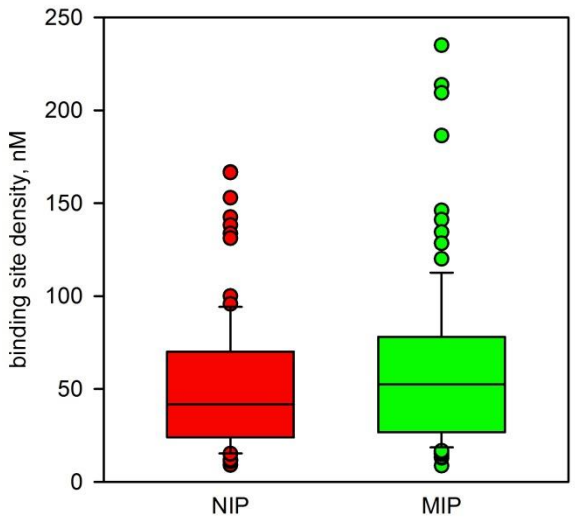


Figure 6. Relationship between the binding site density (B_{max}) values for naproxen measured on the MIP- and NIP-libraries. The blue line indicates the linear regression model of B_{max}(MIP) *vs.* B_{max}(NIP). The black line represents the upper edge for the B_{max}(MIP) < B_{max}(NIP) region.

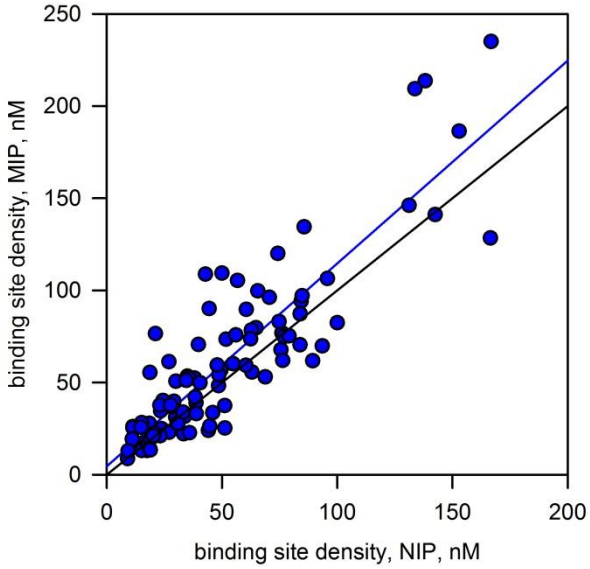


Figure 7. Comparison of apparent affinity constant (K_{eq}) values measured for ibuprofen on the (naproxen-imprinted)MIP- and NIP-libraries applying a Mann-Whitney rank sum test.

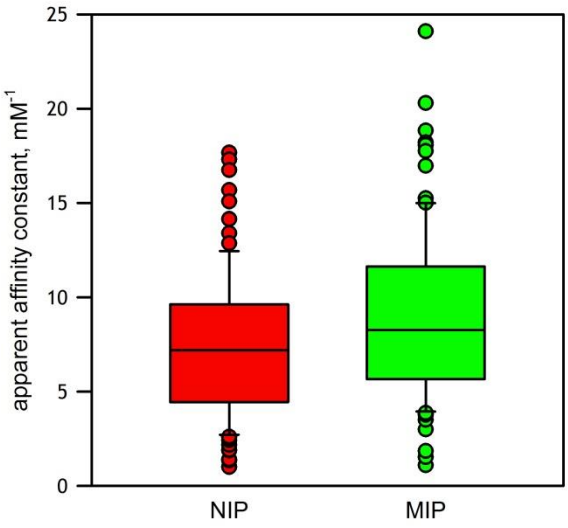


Figure 8. Comparison of the binding site density (B_{max}) values measured for ibuprofen on the (naproxen-imprinted)MIP- and NIP-libraries applying a Mann-Whitney rank sum test.

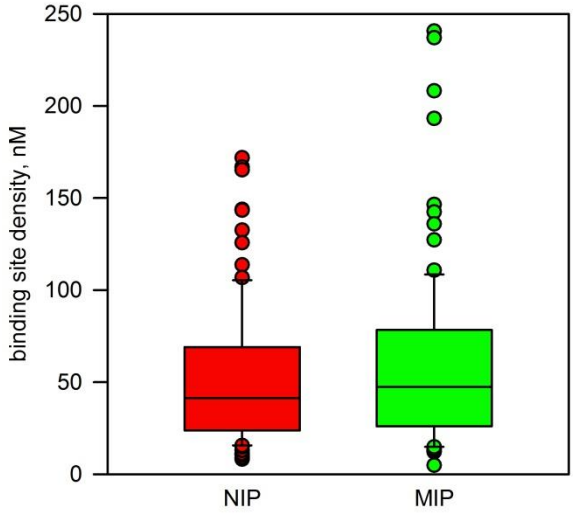
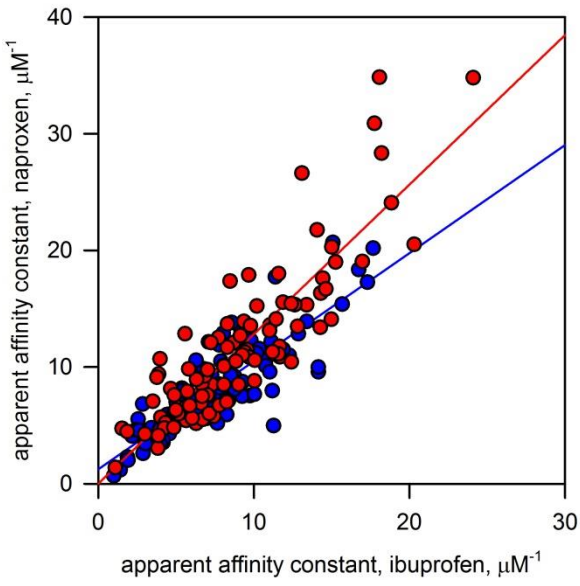


Figure 9. Relationships between the apparent affinity constant (K_{eq}) values measured for ibuprofen and naproxen on the MIP- (red circles) and NIP-libraries (blue circles). The continuous lines indicate the linear regression models for K_{eq}(ibuprofen) *vs.* K_{eq}(naproxen) calculated for NIP- (blue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



ASSOCIATED CONTENT

Supporting Information. Materials and methods, template structures, 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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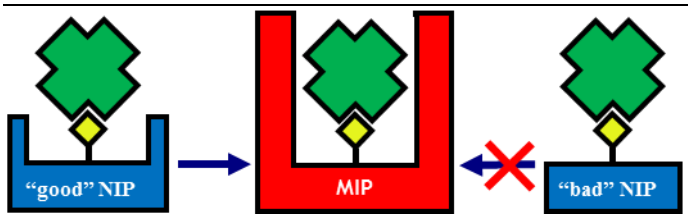
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