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

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EXPERIMENTAL SECTION

Materials. Bisphenol A (BPA), chloramphenicol (CAP), cortisol (COR), diclofenac (DIC), ibuprofen (IBU), metribuzin (MET), naproxen (NAP), pyrimethanil (PYR), theophylline (THO), 2,2-dimethoxy-2-phenylacetophenone (DMPA), all the functional monomers (acrylamide, AM; 2-hydroxyethyl methacrylate, HEMA; methacrylic acid, MAA; 4-vinylpyridine, 4VP), cross-linkers (divinylbenzene, DVB; ethylene dimethacrylate, EDMA; glycerol dimethacrylate, GDMA; pentaerithrytole tetraacrylate, PETA; pentaerithrytole triacrylate, PETRA; trimethylolpropane trimethacrylate, TRIM) were from Sigma–Aldrich–Fluka (Milan, Italy). Acetic acid and all the organic solvents were from VWR International (Milan, Italy). When necessary, polymerization inhibitors in monomers were removed by cleanup on activated alumina columns. Ligands stock solutions were prepared by dissolving 20.0 mg of substance in 4.00 ml of acetonitrile and stored in the dark at −20 °C. All the solvents were of HPLC quality, other chemicals were of analytical grade. For the libraries screening, MultiScreen Resist Filtration System, vacuum manifold and MultiScreen 12×8 wells filter microplates with hydrophilic PTFE membrane were purchased from Millipore (Billerica, USA). The SPE vacuum manifold was from Macherey-Nagel (Duren, Germany), whereas the SPE empty polypropylene columns were from Alltech (Milan, Italy). The HPLC apparatus was an Accela High Speed LC consisting of a 1250 quaternary solvent delivery pump, a thermostated autosampler provided with 25 µl injection system, a diode array UV-Vis detector and a data acquisition system Xcalibur 2.0 from ThermoScientific (Milan, Italy).

Polymeric combinatorial libraries. The polymeric combinatorial libraries were prepared starting from 96 different polymer combinations reported in the supporting informations (table S2). In 3 ml thick wall borosilicate glass vials, prepolymerization solutions with a molar ratio template:functional monomer:cross-linker 1:3:27 were prepared by dissolving 0.05 mmoles of template in the porogenic solvent. When the non-imprinted polymers were prepared, the template was substituted by 11 µl of porogenic solvent. Then, 0.15 mmoles of functional monomer, 1.35 mmoles of cross-linker and 100 µl of a solution of DMPA (1% of the vinyl groups present in the prepolymerization mixture) were added to a proper amount of dry porogenic solvent. The vials were sonicated in an ultrasonic bath for 10 minutes, sealed, and the mixtures were photopolymerized at 4 °C overnight using a 200 W medium-pressure mercury lamp. The bulk polymers obtained were broken with a steel spatula, ground in a mechanical mortar and mechanically wet-sieved to 15–38 µm. The template molecule was extracted from the imprinted combinatorial library by packing the polymers in polypropylene SPE columns and exhaustively washing with acetic acid–methanol 1:9 (v/v) till no template was detectable by the HPLC analysis of the eluate. No efforts were made to measure the amount of template molecule recovered. The same treatment was made in a single washing step for the non-imprinted polymers. The washed polymers were dried under vacuum at 70 °C for 2 h, transferred to glass vials and stored at room temperature. Finally, amounts of about 10 mg of imprinted or non-imprinted polymers were exactly weighed in the wells of an 8x12 filtration microplate in accordance with the scheme reported in the supporting information (table S1). Then the microplates were sealed and stored at room temperature. Later, any binding assay microplates were washed sequentially with 5 x 300 µl of acetic acid–methanol 1:9 (v/v) and 5 x 300 µl of acetonitrile and dried under a gentle stream for 2 h.

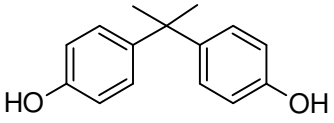
Ligand binding measurements. To measure the ligand binding through a single-point binding assay, 200 µl of 50 µg/ml ligand solutions in acetonitrile were added to the microplate wells, while binding isotherms for naproxen and ibuprofen were measured by adding 200 µl of 5, 10, 15, 25, 50, 75 and 100 µg/ml ligand solutions in acetonitrile to the microplate wells. Then, the microplate was incubated overnight at 4 °C in a TLC chamber saturated with acetonitrile. The ligand solution was filtered, manually transferred into 100-µl HPLC autosampler vials and the free fraction of the ligand was measured by HPLC (*vide infra*). To evaluate the reproducibility of the binding assay, each partition was repeated three times and the amount of free ligand was evaluated as the average of the single values measured. Statistic tests on the ligand binding were performed by using the statistic module of SigmaPlot 11.0 (Systat Software Inc., Richmond, CA, USA), while the calculation of the binding isotherms was performed by using TableCurve 2D 5.0 (Systat Software Inc., Richmond, CA, USA). Non-linear least square fitting was applied to the averaged experimental data by using the Langmuir isotherm model:

$$B = \frac{B_{\max} K_{eq} F}{1 + K_{eq} F} \qquad (1)$$

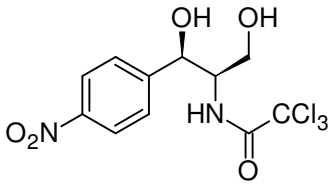
where B is the amount of ligand bound to the polymer, F is the amount of ligand not bound to the polymer, Keq is the apparent affinity constant and Bmax is the binding site density. To assure robust results, Pearson VII limit minimization was chosen as the minimization method. To avoid being trapped in local minima which give incorrect results, the fitting was carried out several times using different initial guess values for the isotherm parameters. The calculated parameters of the isotherm model (p) were not considered significant and the fitting was rejected when the associated standard error (s) did not satisfy the $p > 2s$ constraint.

HPLC analysis. Reverse phase HPLC analysis was used for quantification of the free ligand. Chromatographic separation was performed on a Hypersil Gold reverse-phase C18 (50×2.1 mm) from Thermo Scientific (Milan, Italy). The mobile phase consisted of acidified (0.5% v/v acetic acid) methanol–water 50+50 (v/v) for theophylline (λ=272 nm), 56+44 (v/v) for ibuprofen (λ=221 nm), 60+40 (v/v) for cortisol (λ=254 nm), 65+35 (v/v) for metribuzin (λ=296 nm) and 70+30 for bisphenol A (λ=278 nm), chloramphenicol (λ=276 nm), diclofenac (λ=277 nm), naproxen (λ=233 nm) and pyrimethanil (λ=270 nm). The mobile phase flow-rate was set to 0.50 ml/min. Reference standard solutions for ligands of concentration 0.1, 0.25, 0.5, 1, 2.5, 5 and 10 µg/ml were analyzed three times consecutively and peak areas were plotted against concentration. A calibration curve was drawn using weighted linear regression (weight = 1/conc.). The amount of ligand bound to the polymer was calculated by subtracting the concentration of free ligand from the known initial concentration.

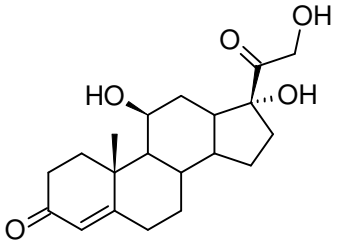
Chart S1: template structures



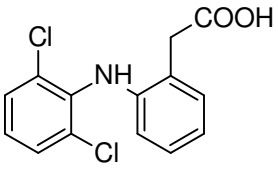
bisphenol A, BPA
logP 3.32, pK 9.78



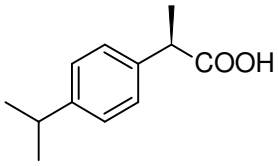
chloramphenicol, CAP
logP 1.15



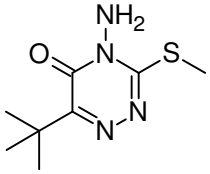
cortisol, COR
logP 1.79



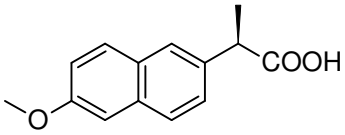
diclofenac, DIC
logP 4.98



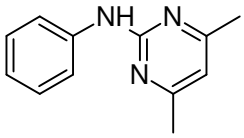
ibuprofen, IBU
logP 3.21, pK 4.80



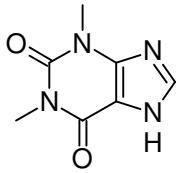
metribuzin, MET
logP 1.70, pK 11.54



naproxen, NAP
logP 3.18, pK 4.19



pyrimethanil, PYR
logP 2.84, pK 10.04



theophylline, THO
logP -0.02, pK 7.82

Table S1: functional monomers, cross-linker and porogenic solvents used to prepare polymeric libraries. AM: acrylamide, HEMA: 2-hydroxyethylmethacrylate, MAA: methacrylic acid, 4VP: 4-vinylpyridine, DVB: divinylbenzene, EDMA: ethylene dimethacrylate, GDMA: glycerol dimethacrylate, PETA: pentaerithrytole tetraacrylate, PETRA: pentaerithrytole triacrylate, TRIM: trimethylolpropane trimethacrylate, MeCN: acetonitrile, $CHCl_3$: chloroform, THF: tetrahydrofurane, TOL: toluene.

		EDMA	DVB	GDMA	TRIM	PETA	PETRA	EDMA	DVB	GDMA	TRIM	PETA	PETRA		
		01	02	03	04	05	06	07	08	09	10	11	12		
MAA	A	MAA 12.7 µl EDMA 255 µl TOL 268 µl	MAA 12.7 µl DVB 192 µl TOL 205 µl	MAA 12.7 µl GDMA 275 µl TOL 288 µl	MAA 12.7 µl TRIM 431 µl TOL 445 µl	MAA 12.7 µl PETA 341 µl TOL 354 µl	MAA 12.7 µl PETRA 436 µl TOL 449 µl	MAA 12.7 µl EDMA 255 µl $CHCl_3$ 268 µl	MAA 12.7 µl DVB 192 µl $CHCl_3$ 205 µl	MAA 12.7 µl GDMA 275 µl $CHCl_3$ 288 µl	MAA 12.7 µl TRIM 431 µl $CHCl_3$ 445 µl	MAA 12.7 µl PETA 341 µl $CHCl_3$ 354 µl	MAA 12.7 µl PETRA 436 µl $CHCl_3$ 449 µl	A	MAA
AM	B	AM 10.6 mg EDMA 255 µl TOL 266 µl	AM 10.6 mg DVB 192 µl TOL 203 µl	AM 10.6 mg GDMA 275 µl TOL 286 µl	AM 10.6 mg TRIM 431 µl TOL 442 µl	AM 10.6 mg PETA 341 µl TOL 341 µl	AM 10.6 mg PETRA 436 µl TOL 447 µl	AM 10.6 mg EDMA 255 µl $CHCl_3$ 266 µl	AM 10.6 mg DVB 192 µl $CHCl_3$ 203 µl	AM 10.6 mg GDMA 275 µl $CHCl_3$ 286 µl	AM 10.6 mg TRIM 431 µl $CHCl_3$ 442 µl	AM 10.6 mg PETA 341 µl $CHCl_3$ 341 µl	AM 10.6 mg PETRA 436 µl $CHCl_3$ 4476 µl	B	AM
4VP	C	4VP 16.2 µl EDMA 255 µl TOL 272 µl	4VP 16.2 µl DVB 192 µl TOL 198 µl	4VP 16.2 µl GDMA 275 µl TOL 291 µl	4VP 16.2 µl TRIM 431 µl TOL 447 µl	4VP 16.2 µl PETA 341 µl TOL 357 µl	4VP 16.2 µl PETRA 436 µl TOL 452 µl	4VP 16.2 µl EDMA 255 µl $CHCl_3$ 272 µl	4VP 16.2 µl DVB 192 µl $CHCl_3$ 198 µl	4VP 16.2 µl) GDMA 275 µl $CHCl_3$ 291 µl	4VP 16.2 µl TRIM 431 µl $CHCl_3$ 447 µl	4VP 16.2 µl PETA 341 µl $CHCl_3$ 357 µl	4VP 16.2 µl PETRA 436 µl $CHCl_3$ 452 µl	C	4VP
HEMA	D	HEMA 18.2 µl EDMA 255 µl TOL 273 µl	HEMA 18.2 µl DVB 192 µl TOL 210 µl	HEMA 18.2 µl GDMA 275 µl TOL 293 µl	HEMA 18.2 µl TRIM 431 µl TOL 449 µl	HEMA 18.2 µl PETA 341 µl TOL 359 µl	HEMA 18.2 µl PETRA 436 µl TOL 454 µl	HEMA 18.2 µl EDMA 255 µl $CHCl_3$ 273 µl	HEMA 18.2 µl DVB 192 µl $CHCl_3$ 210 µl	HEMA 18.2 µl GDMA 275 µl $CHCl_3$ 293 µl	HEMA 18.2 µl TRIM 431 µl $CHCl_3$ 449 µl	HEMA 18.2 µl PETA 341 µl $CHCl_3$ 359 µl	HEMA 18.2 µl PETRA 436 µl $CHCl_3$ 454 µl	D	HEMA

toluene

chloroform

acetonitrile

MAA	E	MAA 12.7 µl EDMA 255 µl TOL 268 µl	MAA 12.7 µl DVB 192 µl TOL 205 µl	MAA 12.7 µl GDMA 275 µl TOL 288 µl	MAA 12.7 µl TRIM 431 µl TOL 445 µl	MAA 12.7 µl PETA 341 µl TOL 354 µl	MAA 12.7 µl PETRA 436 µl TOL 449 µl	MAA 12.7 µl EDMA 255 µl $CHCl_3$ 268 µl	MAA 12.7 µl DVB 192 µl $CHCl_3$ 205 µl	MAA 12.7 µl GDMA 275 µl $CHCl_3$ 288 µl	MAA 12.7 µl TRIM 431 µl $CHCl_3$ 445 µl	MAA 12.7 µl PETA 341 µl $CHCl_3$ 354 µl	MAA 12.7 µl PETRA 436 µl $CHCl_3$ 449 µl	E	MAA
AM	F	AM 10.6 mg EDMA 255 µl TOL 266 µl	AM 10.6 mg DVB 192 µl TOL 203 µl	AM 10.6 mg GDMA 275 µl TOL 286 µl	AM 10.6 mg TRIM 431 µl TOL 442 µl	AM 10.6 mg PETA 341 µl TOL 341 µl	AM 10.6 mg PETRA 436 µl TOL 447 µl	AM 10.6 mg EDMA 255 µl $CHCl_3$ 266 µl	AM 10.6 mg DVB 192 µl $CHCl_3$ 203 µl	AM 10.6 mg GDMA 275 µl $CHCl_3$ 286 µl	AM 10.6 mg TRIM 431 µl $CHCl_3$ 442 µl	AM 10.6 mg PETA 341 µl $CHCl_3$ 341 µl	AM 10.6 mg PETRA 436 µl $CHCl_3$ 4476 µl	F	AM
4VP	G	4VP 16.2 µl EDMA 255 µl TOL 272 µl	4VP 16.2 µl DVB 192 µl TOL 198 µl	4VP 16.2 µl GDMA 275 µl TOL 291 µl	4VP 16.2 µl TRIM 431 µl TOL 447 µl	4VP 16.2 µl PETA 341 µl TOL 357 µl	4VP 16.2 µl PETRA 436 µl TOL 452 µl	4VP 16.2 µl EDMA 255 µl $CHCl_3$ 272 µl	4VP 16.2 µl DVB 192 µl $CHCl_3$ 198 µl	4VP 16.2 µl) GDMA 275 µl $CHCl_3$ 291 µl	4VP 16.2 µl TRIM 431 µl $CHCl_3$ 447 µl	4VP 16.2 µl PETA 341 µl $CHCl_3$ 357 µl	4VP 16.2 µl PETRA 436 µl $CHCl_3$ 452 µl	G	4VP
HEMA	H	HEMA 18.2 µl EDMA 255 µl TOL 273 µl	HEMA 18.2 µl DVB 192 µl TOL 210 µl	HEMA 18.2 µl GDMA 275 µl TOL 293 µl	HEMA 18.2 µl TRIM 431 µl TOL 449 µl	HEMA 18.2 µl PETA 341 µl TOL 359 µl	HEMA 18.2 µl PETRA 436 µl TOL 454 µl	HEMA 18.2 µl EDMA 255 µl $CHCl_3$ 273 µl	HEMA 18.2 µl DVB 192 µl $CHCl_3$ 210 µl	HEMA 18.2 µl GDMA 275 µl $CHCl_3$ 293 µl	HEMA 18.2 µl TRIM 431 µl $CHCl_3$ 449 µl	HEMA 18.2 µl PETA 341 µl $CHCl_3$ 359 µl	HEMA 18.2 µl PETRA 436 µl $CHCl_3$ 454 µl	H	HEMA
		01 EDMA	02 DVB	03 GDMA	04 TRIM	05 PETA	06 PETRA	07 EDMA	08 DVB	09 GDMA	10 TRIM	11 PETA	12 PETRA		

tetrahydrofuran

Note S1: Box plots graphically depict the numerical data considered in this work (bound-to-free values, apparent affinity constants and binding site densities) through their descriptive statistics: the smallest observed, lower quartile (25^{th} percentile of data), median (50^{th} percentile of data), upper quartile (75^{th} percentile of data) and largest observed or calculated data. It shows differences between data populations without making any assumptions of the underlying statistical distribution. Thus, it is suitable to represent both parametric and non-parametric statistical distributions. In the figures, the upper and lower solid boundaries of the boxes indicate the 25th and 75th percentiles, the lines within the boxes mark the medians (50th percentiles), whiskers above and below the boxes indicate the 90th and 10th percentiles, circles above and below whiskers experimental or calculated values external to the 10-90th percentile range, potentially identifying outlyer data. Spacings between the different parts of the box are related to the degree of dispersion and skewness in the data structures.