Artificial antibodies to corticosteroids prepared by molecular imprinting

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Background: Molecular imprinting can be used to prepare antibody and receptor mimics. We have previously shown that acrylic acid polymers can be imprinted to recognize a variety of small molecules. Here, we show that molecularly imprinted polymers (MIPs) can selectively recognize steroid structures.

Results: Artificial antibodies mimicking the binding performance of natural anti-corticosteroid antibodies have been prepared using a molecular imprinting protocol with either cortisol or corticosterone as a target molecule. The binding characteristics of a range of structurally related ligands were estimated using a form of radioimmunoassay. The antibody mimics were found to be highly selective for the ligands used in their preparation and the cross-reactivities with compounds of related structure resembled those obtained in studies with natural antibodies.

Conclusions: The binding properties of MIPs, prepared against corticosteroids, exhibit strong similarities to those of naturally raised antibodies. Such artificial antibodies may serve as a useful complement to their natural counterparts in studies of corticosteroid binding events.

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Introduction

The creation of artificial counterparts to natural macromolecular binding molecules such as proteins is of major interest for many applications. It may be possible to create molecules with the binding properties of natural macromolecules that can be used in harsh environments such as high temperatures and pressures (e.g., sterilization conditions). It is, however, difficult to reproduce the efficiency and selectivity of, for example, receptors interacting with agonists and antagonists, or antibodies recognizing antigens [1]. In addition to other approaches, such as the sophisticated procedures used in the field of supramolecular chemistry [2], molecular imprinting offers a means to produce synthetic molecules that mimic the binding properties of natural macromolecules.

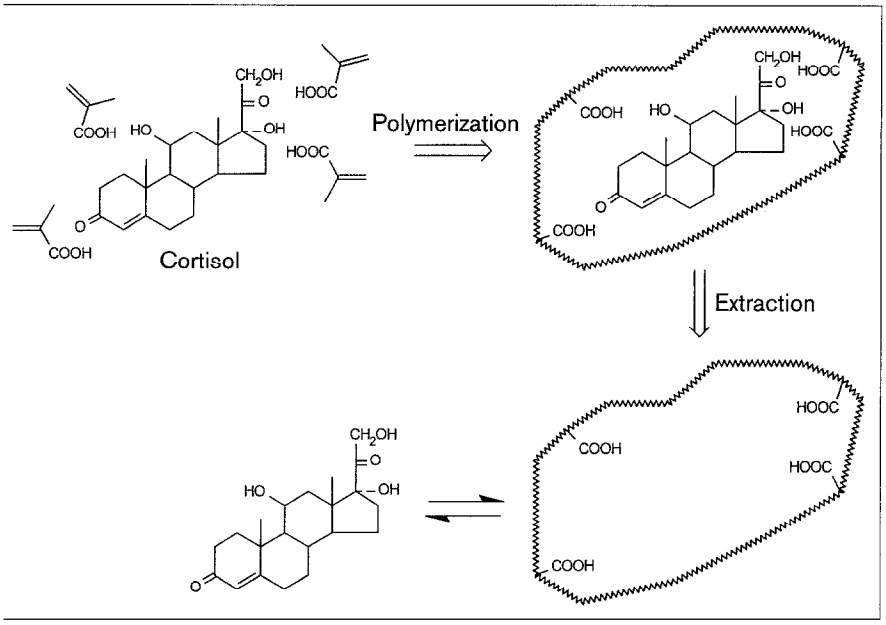
The rapidly expanding field of molecular imprinting is based on the concept of designing recognition sites in macromolecular matrices by means of template polymerization [3–7]. Molecularly imprinted polymers (MIPs) have been shown to possess remarkable recognition properties that have been used in various applications such as drug separations [8–10], template-assisted synthesis [11] and catalysis [12,13], and as receptor mimics [14–17], biomimetic sensors [18] and antibody mimics [19]. The concept is described in Figure 1, where cortisol is shown as the template or print molecule.

The corticosteroids (see Fig. 2) are produced in the adrenal cortex and have widespread effects *in vivo*, including anti-inflammatory action and influences on metabolism, electrolyte and water balance, and various functions of the nervous system [20]. Many medical analyses of corticosteroids, for example those used in assessing the functional status of the adrenal cortex, use antibody-based assay methods such as radioimmunoassay (RIA) and enzyme-linked immunosorbent assay (ELISA) for the selective recognition of a particular corticosteroid [21]. Corticosteroids are potentially very useful for the study of molecular-recognition phenomena [22], and there are a multitude of steroids with very similar structures. The rigid structure of the fused ring system leads to a minimal number of conformations that the molecules may adopt in their interactions with recognition matrices, resulting in a low loss of entropy on binding and, thus, a higher binding strength [23]. Molecular imprinting is highly dependent on non-covalent polar interactions, however, and the number of points capable of such interactions in steroids is small. MIPs against steroids have previously only been produced using strong covalent binding interactions such as carboxylic esters and carbonic acid esters [11,24].

Mimics of natural steroid-binding molecules may be useful for biotechnological applications. Many industrial processes, in which steroids are transformed or used as building blocks, rely on naturally occurring catalysts for efficient selectivity in the synthetic steps [25,26]. Given the physical and chemical properties of MIPs, these materials may be useful for such processes.

The development of efficient mimics of natural binding sites is an ongoing project in our laboratory. Here, we use

Figure 1



Schematic drawing of the molecular imprinting procedure. The print molecule (cortisol) is initially dissolved in the porogen (tetrahydrofuran or acetone) and allowed to form non-covalent complexes with the functional monomer (methacrylic acid). Following addition of crosslinker (ethylene glycol dimethacrylate) and initiator (azo-bisisobutyronitrile), these complexes are fixed by polymerization. Finally, the print molecule is extracted by washing and the molecularly imprinted polymer is ready for association/dissociation studies.

molecular imprinting to make polymers whose binding properties mimic those of anti-corticosteroid antibodies. MIPs were prepared against cortisol and corticosterone and the ligand specificities were assayed using a form of radioimmunoassay. We also determined the number of binding sites and the apparent equilibrium binding constants.

Results and discussion

Preparation of MIPs

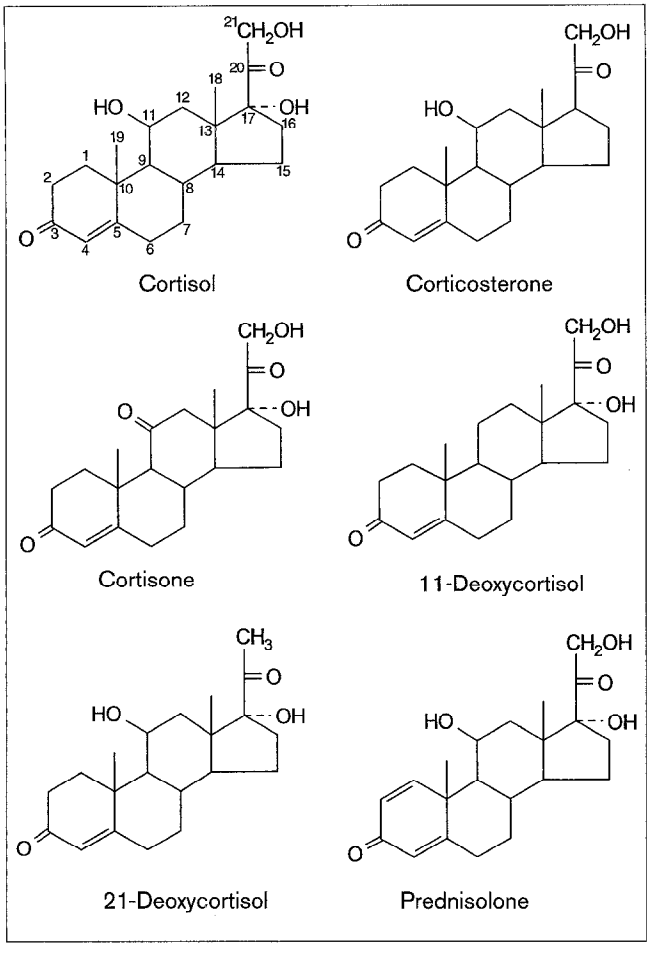
The choice of solvent was important in the self-assembly imprinting protocol used in this study, because only non-covalent interactions are used in the formation and maintenance of the complexes between the functionally active monomers and the print species. More polar solvents had to be used because of the low solubility of the print species in the non-polar solvents, such as dichloromethane and toluene, that are normally used to increase the selectivity of the artificial recognition sites. Thus, two different porogens, tetrahydrofuran and acetone, were tested. The concentrations of the print species used were too high to achieve full solubility, but after addition of functional monomer to a ratio of 10:1 relative to the print molecule, clear solutions were obtained, indicating that strong interactions had been established between the functional monomer and the print species.

Equilibrium binding studies

Once polymerization was completed and the print molecule removed by washing, the capacities of the MIPs for the print species were investigated by saturation of the polymer with increasing amounts of ligand. The assays were performed in several different solvents, but optimal binding performances were achieved with mixtures of tetrahydrofuran and *n*-heptane. To increase the solubility of the ligands for further studies, a small amount of acetic acid was added to the solvent. The resulting imprinting performance, as measured by the saturation studies, was very similar using either imprinting porogen, tetrahydrofuran or acetone. We chose to use tetrahydrofuran, because it more closely resembles the solvent used in the subsequent analyses. The amounts of polymer needed to bind 50 % of the added radiolabelled print species were similar for all of the imprinted polymers: ~1.4 mg for the anti-cortisol polymers (MIP1 and MIP2), and 2.0 mg for the anti-corticosterone polymer (MIP3). The corresponding values for the reference, non-imprinted polymer (REF1) were 6.3 mg and 7.0 mg, respectively. Using a polymer concentration of 1 mg ml^{-1}, the binding to the non-imprinted polymers was 10–16 % of the binding to the imprinted polymers and this concentration level was chosen for further experiments. In the competition protocol used, the amounts of ligand bound to the REF polymers were not sufficiently altered by an increasing amount of competing ligand (in the range studied) to be able to estimate an IC_{50} value. In comparison to the behaviour of the MIPs (MIP1–3; see below), this indicates that the imprinting has been successful, and selective sites have been formed in the MIPs.

The binding characteristics of the polymers are heterogenous in nature, as reflected by non-linear Scatchard plots (Fig. 3). This 'polyclonal' behaviour is an unavoidable effect of the imprinting procedure, in which the weak, non-covalent interactions between the template molecules

Figure 2



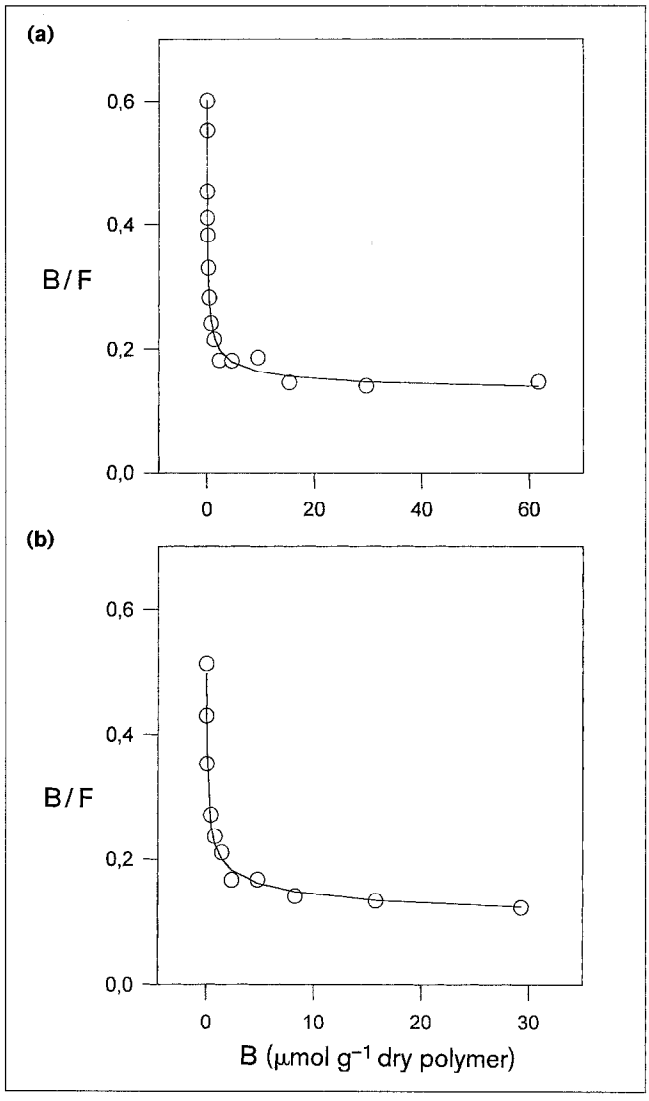
Chemical structures of corticosteroids. Cortisol (hydrocortisone) and corticosterone were used as print molecules.

and the functional monomers lead to the formation of differing sites in the finished polymer. Thus, sites with a wide range of binding affinities are formed: a small number of high affinity sites, and a larger number of sites with low affinity. For the polymers studied, a model that postulates the presence of two types of corticosteroid binding site can be used to describe the binding of the original templates to the imprinted polymers. The values of the dissociation constants and the corresponding binding-site densities for the anti-cortisol and anti-corticosterone polymers are shown in Table 1. The figures are of the same order for both polymers, although the utility of the two-site model is somewhat more pronounced for the anti-cortisol polymer (MIP1), as reflected by a smaller number of sites with higher affinity and a larger density of sites with a lower affinity than is seen for the anti-corticosterone polymer (MIP3). The binding affinities are low compared to natural antibodies, for which dissociation constants from 1–10 nM have been recorded [27,28]. This is, in part, because of the experimental conditions used. If a more sensitive assay method could be used, it might be possible to measure stronger binding affinities for the best sites.

Selectivities of artificial antibodies

The selectivities of the artificial antibodies were estimated by measuring the amount of non-radiolabelled ligand required to displace 50 % of radiolabelled template species from the polymers (IC_{50}). The experimental design is analogous to standard competitive immunoassays, where unlabelled ligands compete with the radiolabelled ligand for binding. Dose–response curves, obtained from experiments in which

Figure 3



The binding characteristics of the imprinted polymers are heterogenous. Scatchard plots for the print species binding to imprinted polymers are shown. B denotes the amount of bound ligand and F is the amount of free ligand. **(a)** Cortisol binding by anti-cortisol polymer MIP1. **(b)** Corticosterone binding by anti-corticosterone polymer MIP3.

ıble 1

›lymer-binding characteristics.

	K_d		B_{max} (μmol g^{-1})	
›lymer	High affinity (x 10^{-6} M)	Low affinity (x 10^{-3} M)	High	Low
IP1	0.57 ± 0.16	1.59 ± 0.73	0.21 ± 0.05	280 ± 120
IP3	1.23 ± 0.43	0.84 ± 0.47	0.37 ± 0.12	130 ± 60

e values are given (with standard deviations) for estimated high- and low-affinity sites calculated according to a two-site model. B_{max} indicates ə density of binding sites in the polymer.

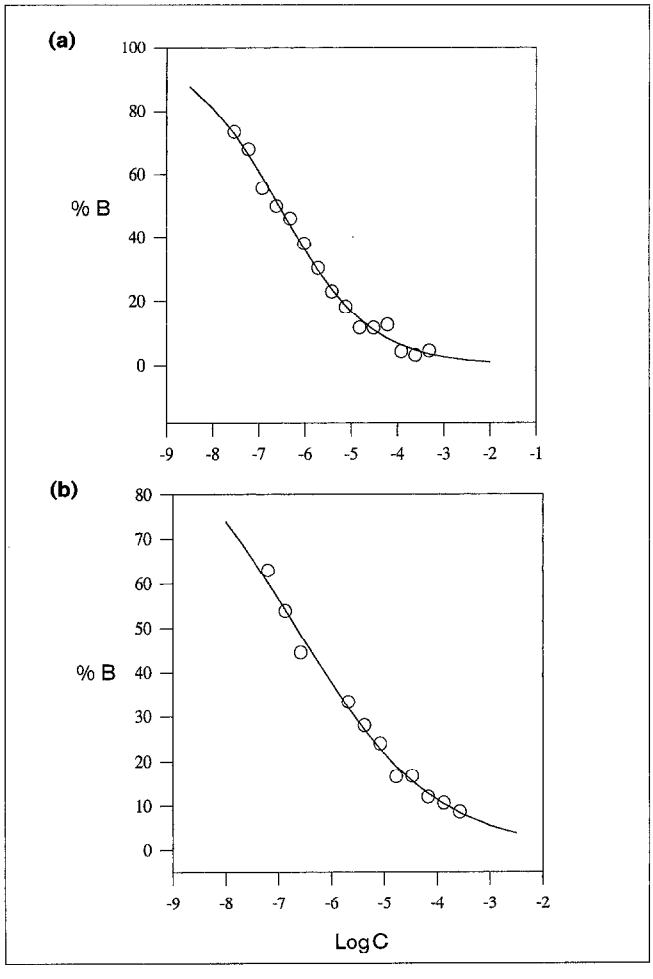
ılabelled print species (cortisol or corticosterone) were used competing ligands against MIP1 or MIP3, are shown in gure 4. The IC_{50} values from the competition assays and e calculated cross-reactivities are presented in Table 2.

'om the structures of the various corticosteroids analyzed ig. 2), the implications of various functionalities for recogtion by MIPs can be deduced. For the anti-cortisol polyər (MIP1), the removal of one hydroxyl functionality from e imprinted structure leads to a decrease in binding as flected by the increased IC_{50} values. Removal of the 11-, - or 21-OH group reduces the binding to MIP1 15-, 12- or -fold, respectively. This reduction can be explained by e loss of hydrogen bonding between the hydroxyl groups the corticosteroids and the carboxyl groups of the rrounding polymer. Changing the 11-OH group for a keto nctionality as in cortisone causes a drastic (>100-fold) ːcrease in binding. This may also be due to the difference the hydrogen bonding capabilities of the two molecules. the cortisol structure the hydroxyl group is able to act as ıth hydrogen donor and hydrogen acceptor, stabilizing the teraction with a carboxyl functionality of the methacrylic id residues of the polymer. The keto functionality of rtisone is unable to act as a hydrogen donor, leading to ːaker interactions. Another explanation for this effect may : the steric constraints of the cortisone structure compared cortisol. The presence of the planar keto functionality in e β-position, as opposed to the hydroxyl group, may lead changes in ring structure that cannot be accommodated the imprinted polymer.

gand binding to MIP1 is not severely affected by the esence of a single unsaturated carbon atom at the Δ-1 ısition, as in prednisolone. The calculated cross-reactivity r this compound is as high as 36 %. The minor locking of e flexibility of the A-ring in this compound results in only slight reduction in binding to MIP1, as it is unable to apt to all of the configurations possible for cortisol.

ɔr the anti-corticosterone polymer, MIP3, introduction an additional hydroxyl group in the 17-α-position ortisol) reduces the binding 10-fold. This is most likely ıe to steric constraints on the fit of the cortisol molecule to the more tightly formed site for corticosterone. The cross-reactivities of the other ligands studied suggest that the anti-corticosterone polymer is more selective than the anti-cortisol polymer. However, apart from cortisol the ligands differ from corticosterone in two positions and,

Figure 4



The cross-reactivities of corticosteroids with MIPs can be estimated using competition studies. The dose–response curves for the binding of the print species to the MIPs are shown. B denotes amount of bound radiolabelled ligand and C is the concentration of competing ligand. **(a)** Cortisol binding by anti-cortisol polymer MIP1. **(b)** Corticosterone binding by anti-corticosterone polymer MIP3.

Table 2

IC_{50} values and cross-reactivities of corticosteroid ligands.

	MIP1[a]		MIP3[b]	
	IC_{50}[c]	Cross-reactivity[d]	IC_{50}[c]	Cross-reactivity[d]
Cortisol	0.27	100	2.2	10
Corticosterone	3.1	8.6	0.22	100
21-Deoxycortisol	6.7	4.0	41	0.54
11-Deoxycortisol	3.9	6.8	13	1.6
Prednisolone	0.74	36	3.8	5.7
Cortisone	30.0	0.89	57	0.38

[a]Anti-cortisol polymer.
[b]Anti-corticosterone polymer.
[c]μM.
[d]Cross-reactivity value is defined as 100 % for the print species.

using cortisol as a reference, the discrimination of these ligands by the anti-corticosterone polymer is less pronounced than by the anti-cortisol polymer. The effects of structural changes to the ligands exhibit clear trends that are similar to those seen with the anti-cortisol polymer. Using cortisol as the reference species, removal of one hydroxyl functionality from the binding species reduces the binding to MIP3 from 6- to 19-fold. Introduction of a double bond in the A-ring (prednisolone) reduces the binding nearly 2-fold, similar to the effect seen with the anti-cortisol polymer. The exchange of the 11-β-OH group for the planar keto group reduces binding 26-fold.

There are strong similarities between the binding properties of the MIPs and those of commercially available antibodies and antibodies reported in the scientific literature (Table 3). The cross-reactivities of the anti-cortisol antibodies are similar to those observed with the anti-cortisol polymer. Natural antibodies are either monoclonal or are subjected to some sort of screening process, resulting in optimized performance. The MIPs have a range of binding sites and can be thought of as 'polyclonal', which will inevitably reduce the binding selectivities.

Sensitivity

With the protocol used in this investigation, the detection limits for cortisol and corticosterone are in the range of 10^{-7}–10^{-8} M using the anti-cortisol and anti-corticosterone polymers, respectively, as indicated by the dose–response curves shown in Figure 4. This limitation is, in part, a consequence of the detection method used, and the values might be improved by developing a more sensitive assay.

To investigate the performance of the polymers in aqueous media, binding assays were performed using several buffer systems. The selectivities of the polymers were severely diminished, however, and only minute differences between binding to the imprinted polymers and the reference polymers could be detected (data not shown). This may be because the interactions between the print species and the functional monomers in the imprinting process consist mainly of hydrogen bonds. When subjected to aqueous buffer, these interactions are disrupted because of the strong hydrogen-bonding capacity of the water molecules. Although antibody-based methods for detection of steroids have been developed for immediate use in aqueous environments, such as plasma or urine samples, several techniques use an extraction step into organic solvents to avoid interferences with, for example, native steroid-binding proteins.The use of anti-corticosteroid MIPs in organic solvents may thus be a viable alternative for analytical applications.

Significance

We have prepared artificial antibodies selective for particular corticosteroids using molecular imprinting.

Table 3

Cross-reactivities of various corticosteroid immunoassays as stated by the manufacturers or authors[a].

	Cortisol assays				Corticosterone assays	
Ligand	ELISA[b]	RIA1[c]	RIA2[d]	RIA3[e]	RIA4[f]	RIA5[g]
Cortisol	100	100	100	100	2.7	0.03
Corticosterone	10	1.0	0.6	3.0	100	100
21-Deoxycortisol	<0.1	8.0	0.3	-	-	-
11-Deoxycortisol	19	-	-	-	-	<0.01
Prednisolone	13	11	46	-	-	-
Cortisone	-	-	-	16	-	-

[a]Values shown are percent cross-reactivity.
[b]Monoclonal mouse anti-cortisol antibodies [29].
[c]Sorin Biomedica, Almere, NL.
[d]Diagnostic Products Corporation, Los Angeles, CA, USA.
[e]Polyclonal rabbit anti-cortisol serum [30].
[f]Polyclonal rat anti-corticosterone serum [27].
[g]ICN Biomedicals, Inc., Costa Mesa, CA, USA.

Artificial antibodies prepared by this method are conceptually attractive complements to their natural counterparts. The advantages of MIPs over natural antibodies and receptors include stability and the ability to perform in harsh conditions such as at extreme pH, under the high temperatures and pressures found under sterilization conditions, or in organic solvents. Molecular imprinting may prove useful for preparing materials that recognize proteins or other biological compounds for which the structural information needed for rational ligand design is lacking. Likewise, if a natural receptor is poorly characterized or difficult to isolate, artificially prepared mimics may serve as a useful complement. Such polymers also are considerably less costly to produce when compared to natural antibodies or receptors.

Material and methods

Chemicals

Cortisol, corticosterone, 21-deoxycortisol, 11-deoxycortisol, prednisolone and cortisone were from Sigma Chemical Co. (St. Louis, MO, USA). [1,2,6,7-^{3}H]cortisol (specific activity 2.22 TBq mmol^{-1}) and [1,2,6,7-^{3}H]corticosterone (specific activity 3.03 TBq mmol^{-1}) were from Amersham International plc. (Little Chalfont, UK). Methacrylic acid (MAA), ethylene glycol dimethacrylate (EDMA) and azo-bisisobutyronitrile were from Merck (Darmstadt, FRG). Scintillation liquid, Ecoscint O, was from National Diagnostics (Manville, NJ, USA). All solvents were of either high pressure liquid chromatography (HPLC) or analytical grade.

Preparation of MIPs

The MIPs were prepared according to Table 4. The print molecule (1.31 mmol) was dissolved in 20 ml dry porogen, either tetrahydrofuran or acetone, together with freshly distilled functional monomer, methacrylic acid (13.1 mmol). The crosslinker, ethylene glycol dimethacrylate (65.6 mmol), and the initiating agent, azo-bisisobutyronitrile (125 mg), were added and the solution was chilled on an ice-bath and purged thoroughly with nitrogen for 10 min. The degassed solution was photolytically polymerized under a nitrogen atmosphere at 4 °C overnight using a standard laboratory UV source at 366 nm (CAMAG, Bubendorf, CH). The resulting polymer was crushed, ground in a mechanical mortar (Retsch, Haan, FRG) and wet-sieved (25 μm, Retsch) with water. Fine particles were removed through repeated sedimentation in acetone. The print species were extracted by extensive washing with methanol:acetic acid (9:1, v/v), followed spectrophotometrically at 242 nm until no more print molecule could be detected.

Equilibrium binding studies

The capacity of the polymers was measured by saturation studies. Radiolabelled ligand (500 Bq) was added to polymer particles ranging in concentration from 0.03 to 20 mg ml^{-1} in a total volume of 1.0 ml solvent in polypropylene microcentrifuge tubes (Brand, Wertheim, FRG). The binding was allowed to reach equilibrium at ambient temperature on a rocking table overnight. The polymer particles were removed from the samples by centrifugation at 10,000g for 5 min, 500 μl of the supernatant was added to 10 ml of scintillation cocktail in 20 ml scintillation vials (National Diagnostics, Atlanta, GA, USA) and the radioactivity was measured using a model 2119 RACKBETA β-radiation counter (LKB Wallac, Sollentuna, Sweden).

Competition studies

The competition assays were performed in a similar way to the binding studies. Non-radiolabelled ligand ranging from 0.01 to 250 μg was mixed with 1.0 mg of polymer particles in polypropylene microcentrifuge tubes. Radiolabelled ligand (500 Bq) was added and the made up to 1.0 ml with solvent. The samples were allov equilibrium overnight at ambient temperature on a rockir sample was centrifuged at 10,000g for 5 min and the amo ligand was estimated by adding 500 μl supernatant scintillation liquid and counting the sample in a β-radiation concentration of ligand capable of displacing 50 % of b (IC_{50}) was calculated using the computer softwa EBDA/LIGAND (Elsevier-Biosoft, Amsterdam, NL). Cros: were calculated as relative IC_{50} values, where the cross the imprint species was set as 100 %.

Table 4

Chemical composition of MIPs.

Polymer	Print molecule	Monomers (Ratio[a])	
MIP1	Cortisol	MAA/EDMA (10:50)	Tetr
MIP2	Cortisol	MAA/EDMA (10:50)	,
MIP3	Corticosterone	MAA/EDMA (10:50)	Tetr
REF1	None	MAA/EDMA	Tetr
REF2	None	MAA/EDMA	,

[a]Molar ratio relative to print molecule.

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