Molecular Recognition

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A Stoichiometric Molecularly Imprinted Polymer for the Class-Selective Recognition of Antibiotics in Aqueous Media**

Javier L. Urraca, Andrew J. Hall, Maria C. Moreno-Bondi, and Börje Sellergren**

Since the introduction of penicillin in 1941, antibiotics have been used effectively in both human and veterinary medicine to fight infectious diseases[1] and, in the latter case, as food additives to promote livestock growth.[2] Antibiotic residues have been observed in aquatic environments, including groundwater, surface water, and tap water, thus raising concern because of both the toxicity of these drugs to aquatic organisms and the emergence of resistant bacterial strains,[3,4] which may impact greatly on the ability of humankind to fight infectious diseases. Also of concern is the presence of antibiotics in meat and dairy products, which can lead to allergic reactions in hypersensitive individuals.[5] Accordingly, the use of antibiotics in veterinary medicine has been restricted or prohibited in some countries,[6] with maximum permitted levels having been defined by both European and North American legislators. Efficient analytical methods are therefore necessary to satisfy the regulatory levels of these compounds in foodstuffs.

In recent years, there has been growing interest in molecularly imprinted polymers (MIPs)[7] and their use as stationary phases for the selective extraction of a variety of molecules, for example, pharmaceuticals, foodstuff components, and pollutants, from a variety of complex matrices, such as blood, urine, and environmental waters.[8] A few reports on the imprinting of antibiotics have appeared,[9–15] with one notable exception:[9] MIPs capable of selectively extracting

[*] Dr. A. J. Hall,[+] Priv.-Doz. B. Sellergren
Institut für Umweltforschung
Universität Dortmund
Otto-Hahn-Strasse 6, 44221 Dortmund (Germany)
Fax: (+49) 231-755-4084
E-mail: andy.hall@sunderland.ac.uk
borje@infu.uni-dortmund.de

Dipl.-Chem. J. L. Urraca, Priv.-Doz. M. C. Moreno-Bondi
Departamento de Química Analítica
Facultad de Ciencias Químicas
Universidad Complutense de Madrid
28040 Madrid (Spain)

[+] Present address:
Institute of Pharmacy, Chemistry and Biomedical Sciences
The University of Sunderland
Wharncliffe Street, Sunderland, SR1 3SD (UK)

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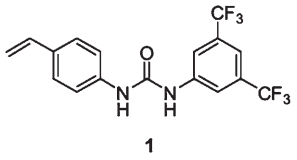
penicillins from aqueous media have not been reported. This omission is because of the common reliance on the conventional noncovalent imprinting method in which the binding sites are templated through weak-to-medium strength interactions.[7] Typically, only a few percent of the added template give rise to imprinted sites, with the remaining noncomplexed functional monomer contributing to nonspecific binding.

This problem can be overcome by the use of designed "host" monomers capable of stoichiometrically interacting with the template functionalities.[9,16–19] With particular emphasis on the imprinting of oxyanions,[17–19] we report herein our studies on the use of one such monomer **1**, based



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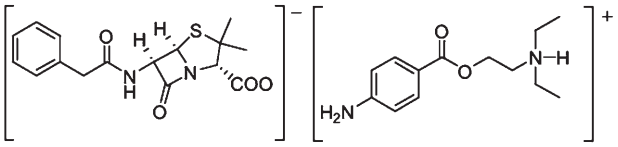


2P

on the urea motif, in the imprinting of penicillin G as its procaine salt **2P**. The association constant previously reported[19] for the interaction between **1** and tetrabutylammonium benzoate ($K_a = 8800\,\text{M}^{-1}$) in dimethyl sulfoxide (DMSO) should be sufficiently high to promote full complexation of **2P** in the prepolymerization mixture.

The polymers were first tested for their ability to retain the template molecule **2P** and the related salt **2K** (Table 1). We chose to evaluate the materials using mixtures of MeCN and aqueous 2-[4-(2-hydroxyethyl)-1-piperazinyl]ethanesulfonic acid (HEPES) buffer (pH 7.5). This pH value was chosen to ensure deprotonation of the carboxylic acid groups of the analytes, which is necessary for binding to occur to the polymeric urea moieties.

Table 1: Retention behavior of **2P** and **2K** on the imprinted and nonimprinted polymers.

Mobile phase (MeCN/ buffer)	k_{MIP} (**2P**)	k_{NIP} (**2P**)	IF (**2P**)	k_{MIP} (**2K**)	k_{NIP} (**2K**)	IF (**2K**)
100:0	8.3	6.1	1.4	n.e.	18.1	>1.9
95:5	0.41	0.16	2.6	12.0	3.0	4.0
90:10	0.41	0.18	2.3	0.93	0.18	5.2
50:50	0.82	0.41	2.0	0.84	0.18	4.7
10:90	**5.0**	**0.47**	**11**	**6.9**	**0.50**	**14**
0:100	31.8	25.0	1.4	n.e.	29.7	>1.15

Sample volume = 20 μL; analyte concentration = 10 mM (**2P** in MeCN, **2K** in MeCN/water (90:10)); flow rate = 1 mL min^{-1}; column dimensions = 125 × 4.6 mm i.d.; detection at $\lambda = 220$, 265 nm; void volume marker was methanol; n.e. = no elution observed over 60 min. The retention factor (k) and imprinting factor (IF) were calculated as $k = t_R - t_0/t_0$, where t_R is the retention time of the analyte, t_0 the retention time of methanol, and $\text{IF} = k_{MIP}/k_{NIP}$. Results shown are the average of three separate analyte injections.

In pure MeCN, we observe an imprinting effect for the template **2P**, though retention on the MIP is only marginally stronger than on the control nonimprinted polymer (NIP). However, **2K** is very strongly retained on the MIP (no elution within 60 minutes) and shows a higher retention on the NIP than **2P** does. These phenomena are possibly related to mass-transport effects in which the more bulky ion pair **2P** is expected to have greater difficulty in diffusing into the binding sites than the smaller **2K**. This explanation is further supported by the higher imprinting factors (IFs) observed for **2P** at higher buffer contents at which the ion pairs are expected to be fully dissociated.

It should be noted that preliminary HPLC experiments using pure water in place of buffer (results not shown) showed that the retention of **2K** was always dramatically stronger than that of the template **2P**, thus prompting us to use HEPES buffer (pH 7.5) in all further experiments.

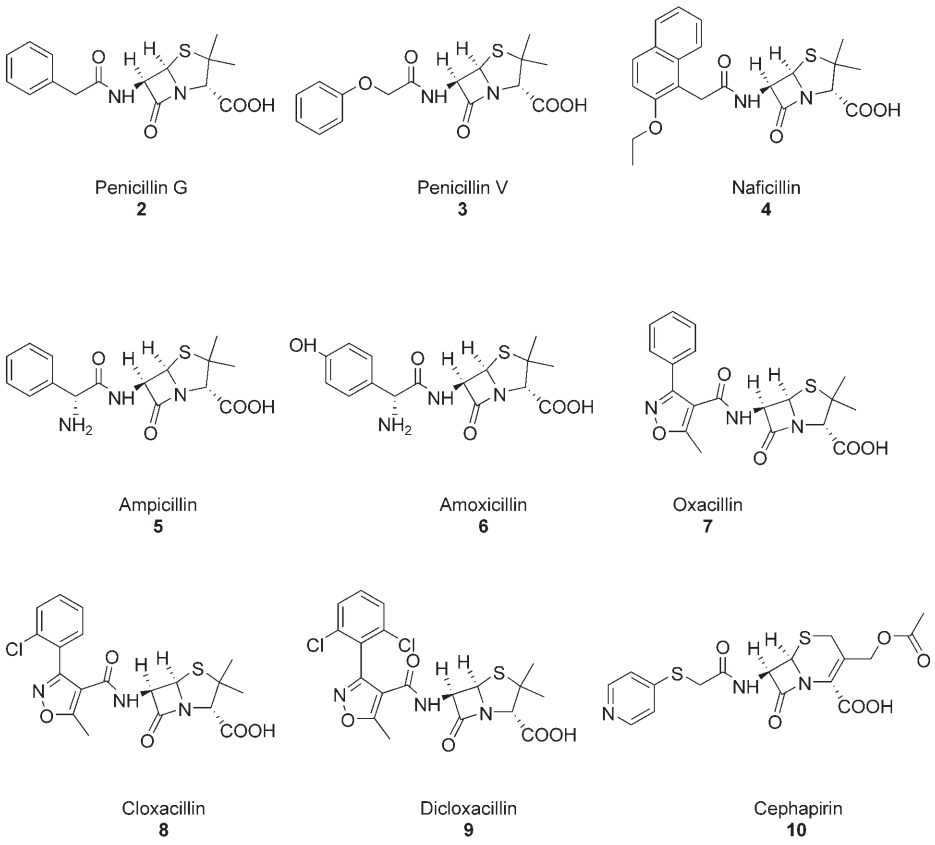
The addition of 5% water leads to a dramatic decrease in the retention times for **2P** on both the MIP and NIP, with a similar effect occurring at 10% buffer for **2K**. However, retention on the MIP remains stronger than on the NIP in all cases, thus leading to significant IFs. At 50% aqueous buffer, there is a slight rise in the retention times, with the analytes again more retained on the MIP than on the NIP.

The most interesting observations occur at 90% buffer. Under these conditions, the retention times of the analytes rise significantly on the MIP but, crucially, not on the NIP. It is in this mobile phase that the difference between the recognition properties of the MIP and NIP is most striking, with an IF of >10 for both salts (Table 1). Finally, the retention times climb exponentially in pure aqueous buffer for both the MIP and NIP, although retention on the MIP remains stronger than on the NIP. This retention behavior is commonly observed for MIPs and has been explained by a shift from an electrostatic retention mode in water-poor mobile phases to a hydrophobic mode in water-rich systems in which both modes involve a specific and a nonspecific contribution to the overall retention.[20,9]

We next decided to test the ability of the imprinted polymer to retain other related analytes, namely β-lactam antibiotics **2**–**10**, with the optimal mobile phase as defined by the experiments described above, that is, MeCN/buffer (10:90, v/v).

As can be seen in Figure 1, the retention of all the β-lactam antibiotic analytes on the MIP is rather strong. The template **2P** and the related salt **2K** are the most strongly retained analytes, further supporting the success of the imprinting step. With the exception of amoxicillin (**6**), the MIP appears to contain binding sites principally for the β-lactam carboxylate structure that are capable of accommodating the pendant substituents. Cephapirin (**10**), with a six-membered ring in the β-lactam structure, is the least-well retained of such antibiotics. Finally, the unrelated compound benzoic acid, used as a control analyte, is retained extremely weakly on the imprinted polymer.

In contrast to the retention behaviors shown on the MIP, all analytes are similarly and weakly retained on the control



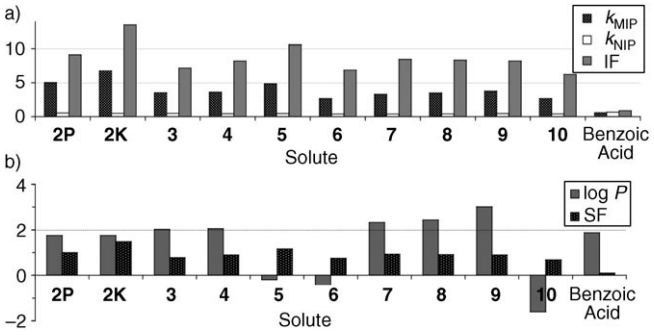


Figure 1. Evaluation of the cross-selectivity of the polymers in terms of a) the retention factors (k) for the MIP, the NIP, and the corresponding imprinting factors (IF) and b) the selectivity factor (SF) and hydrophobicity ($\log P$ values) for the analytes. SF = IF (analyte)/IF (template). Mobile phase = MeCN/HEPES buffer (aq.; pH 7.5; 10:90, v/v), analyte concentration = 1 mM; the void volume marker was methanol. Experimental conditions otherwise as in Table 1.

NIP, again supporting the success of the imprinting step. Attempts were made to correlate the retention data with the physical properties of the analytes used. As is obvious from the lack of correlation between the selectivity factor (SF) and the $\log P$ values shown in Figure 1b,[21] nonspecific hydrophobic retention is not the cause of the enhanced retention observed on the MIP. There appears to be a small trend of increasing retention with increasing $\log P$ values within the series **7**–**9**. However, the retention of the far more hydrophilic zwitterionic analyte **5** shows that this is not a general effect. Instead, we attribute the effects to the existence of complementary binding sites capable of accommodating the β-lactam family of solutes, in which analytes with greater structural similarity to the template molecule display the higher retention factors.

To gain insight into the binding energy and site density of the polymers, we measured the equilibrium binding isotherms for both the imprinted and nonimprinted polymer in the optimum solvent system described above, in which the template is expected to bind strongly to the imprinted polymer (Figure 2). The isotherms showed a good fit to a Langmuir binary site model, thus resulting in two classes of binding sites with largely different binding energies. The resulting parameters gave a maximum binding constant for the MIP of $7800\,\text{M}^{-1}$ and an excess capacity of the MIP over the NIP near the nominal capacity ($112\,\mu\text{mol g}^{-1}$) calculated on the basis of the amount of added template to the prepolymerization mixture. This behavior shows that the matrix urea groups are functional and fully accessible. In the case of the NIP, the high-energy sites have a similar association constant to those of the MIP, although the capacity is much lower, whereas the low-energy sites are weaker and less

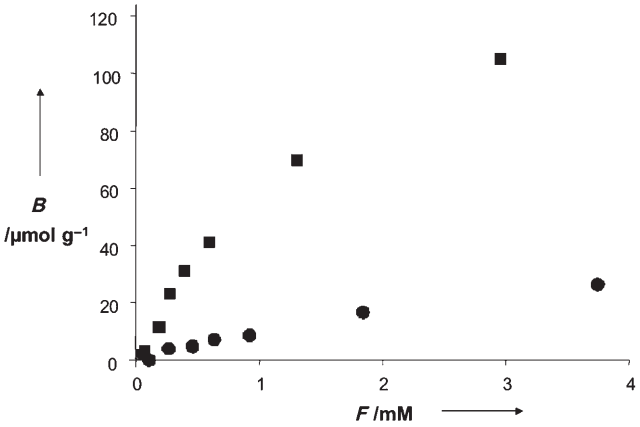


Figure 2. Equilibrium binding isotherms for the uptake of the penicillin G procaine salt by MIP (squares) and NIP (circles) in MeCN/aq. HEPES buffer (pH 7.5; 10:90, v/v). F = concentration of the free solute, B = specific amount of bound solute. The binding constants (K_a) and specific binding capacities (N) were obtained from linear fitting of corresponding Scatchard plots to a Langmuir binary site model. The fitting parameters for the class 1 and 2 sites of the MIP were: 1) $K_a = 7780\,\text{M}^{-1}$, $N = 36.6\,\mu\text{mol g}^{-1}$ ($R^2 = 0.99$); 2) $K_a = 610\,\text{M}^{-1}$, $N = 163\,\mu\text{mol g}^{-1}$ ($R^2 = 0.98$); and for the NIP: 1) $K_a = 7080\,\text{M}^{-1}$, $N = 7.3\,\mu\text{mol g}^{-1}$ ($R^2 = 0.98$); 2) $K_a = 240\,\text{M}^{-1}$, $N = 57.4\,\mu\text{mol g}^{-1}$ ($R^2 = 0.87$). Each data point represents the average of two replicate measurements with a coefficient of variation in the range of 2.7–5.6%.

prevalent than those in the MIP. These results suggest that the differences in retention observed during the chromatographic testing relate more to the number than the binding energy of the high-affinity sites present.

In summary, a stoichiometrically imprinted polymeric receptor for the class-selective recognition of β-lactam antibiotics has been prepared and shown to effectively retain such compounds under predominantly aqueous conditions. We are currently assessing the use of these materials for solid-phase extraction (SPE) in the selective removal of β-lactam antibiotics from environmental water samples and as receptors for fluorescence based assays.

Experimental Section

Polymer preparation: The template **2P** (286 mg, 0.5 mmol), functional monomer **1** (186 mg, 0.5 mmol), methacrylamide (84 mg, 1 mmol), ethylene glycol dimethacrylate (EDMA; 3.8 mL, 20 mmol), and the free radical initiator 2,2′-azo-bis-(2,4-dimethylvaleronitrile) (ABDV; 44 mg, 1 % w/w total monomers) were dissolved in MeCN (5.6 mL). The solution was transferred to a glass tube, cooled to 0 °C, and purged with N_2 for 10 min to remove dissolved oxygen. After purging, the glass tube was sealed and polymerization initiated thermally by placing the tube in a water bath set at 40 °C. Polymerization was allowed to proceed at this temperature for 48 h. The tube was then broken, and the MIP monolith was removed and broken into smaller fragments. The template was removed through sequential washing with MeOH (100 mL), MeOH/0.1M HCl (9:1, 100 mL), and finally MeOH (100 mL).

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[1] A. M. , Johnston, *Int. J. Antimicrob. Agents* **2001**, *18*, 291 – 294.
[2] P. Boerlin, A. Wissing, F. M. Aarestrup, J. Frey, J. Nicolet, *J. Clin. Microbiol.* **2001**, 4193 – 4195.
[3] H. J. Stan, T. H. Heberer, M. Linkerhägner, *Vom Wasser* **1994**, *83*, 57 – 68.
[4] V. H. Smith, F. DeNoyelles, Jr., C. K. Larive, *Environ. Sci. Technol.* **2003**, *37*, 1713 – 1719.
[5] G. Patriarca, C. D'Ambrosio, D. Schiavino, E. Nucera, J. Y. Sun, M. Del Ninno, A. Buonomo, *Int. Arch. Allergy Immunol.* **1999**, *118*, 247 – 250.
[6] B. Hileman, *Chem. Eng. News* **2001**, *79*(8), 47 – 52.
[7] a) C. Alexander, H. S. Andersson, L. I. Andersson, R. J. Ansell, N. Kirsch, I. A. Nicholls, J. O'Mahony, M. J. Whitcombe, *J. Mol. Recognit.* **2006**, *19*, 106 – 180; b) (Ed.: B. Sellergren), *Molecularly Imprinted Polymers: Man-Made Mimics of Antibodies and their Application in Analytical Chemistry.* Techniques and instrumentation in analytical chemistry, Elsevier Science, Amsterdam, Netherlands, **2001**.
[8] F. Lanza, B. Sellergren, *Chromatographia* **2001**, *53*, 599 – 611.
[9] C. Lübke, M. Lübke, M. J. Whitcombe, E. N. Vulfson, *Macromolecules* **2000**, *33*, 5098 – 5105.
[10] J. Cederfur, Y. Pei, M. Zihui, M. Kempe, *J. Comb. Chem.* **2003**, *5*, 67 – 72.
[11] K. Skudar, O. Brüggemann, A. Wittelsberger, O. Ramström, *Anal. Commun.* **1999**, *36*, 327 – 331.
[12] R. Levi, S. McNiven, S. A. Piletsky, S.-H. Cheong, K. Yano, I. Karube, *Anal. Chem.* **1997**, *69*, 2017 – 2021.
[13] M. Senholdt, M. Siemann, K. Mosbach, L. I. Andersson, *Anal. Lett.* **1997**, *30*, 1809 – 1821.
[14] M. Siemann, L. I. Andersson, K. Mosbach, *J. Antibiot.* **1997**, *50*, 89 – 91.
[15] E. Benito-Pena, M. C. Moreno-Bondi, S. Aparicio, G. Orellana, J. Cederfur, M. Kempe, *Anal. Chem.* **2006**, *78*, 2019 – 2027.
[16] G. Wulff, K. Knorr, *Bioseparation* **2002**, *10*, 257 – 276.
[17] A. J. Hall, L. Achilli, P. Manesiotis, M. Quaglia, E. De Lorenzi, B. Sellergren, *J. Org. Chem.* **2003**, *68*, 9132 – 9135.
[18] P. Manesiotis, A. J. Hall, M. Emgenbroich, M. Quaglia, E. De Lorenzi, B. Sellergren, *Chem. Commun.* **2004**, 2278 – 2279.
[19] A. J. Hall, P. Manesiotis, M. Emgenbroich, M. Quaglia, E. De Lorenzi, B. Sellergren, *J. Org. Chem.* **2005**, *70*, 1732 – 1736.
[20] a) C. Dauwe, B. Sellergren, *J. Chromatogr.* **1996**, *753*, 191; b) J. G. Karlsson, L. I. Andersson, I. Nicholls, *Anal. Chim. Acta* **2001**, *435*, 57 – 64.
[21] Log *P* values were calculated using ChemOffice ChemDraw Pro V 9.0.1 software.

