Bioorganic Chemistry

An Artificial Riboflavin Receptor Prepared by a Template Analogue Imprinting Strategy**

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Molecular imprinting has resulted in a range of robust polymer-based receptors that are being considered for use in a variety of applications based on molecular recognition.[1] The technique entails polymerization of mono- and polyfunctional monomers in the presence of a template, whose subsequent removal leaves sites that can be reoccupied by the template or a closely related compound. These synthetic receptors are distinguished by their robustness and ease of synthesis, but they also have drawbacks, notably their poor water compatibility and, with notable exceptions,[2–5] the lack of strategies for imprinting water-soluble target molecules. One approach towards overcoming the latter is to use a non-aqueous imprinting protocol, in which lipophilic templates representing a close structural analogue or substructure of the target are employed.[6,7] Here, a solvent/porogen is chosen such that the intrinsically weak monomer–template interactions based, for example, on hydrogen bonding, electrostatics, or charge transfer, are stabilized. Thus, the use of solvents with low polarity and hydrogen bonding strength is generally favored. Although polymers prepared by this route have displayed selective binding of their targets under aqueous conditions, the effects were typically too weak to be of practical value. Following this approach, we report here on molecularly imprinted polymers (MIPs) that recognize their targets (riboflavin) effectively under such conditions (Figure 1). Careful fine-tuning of the synthesis conditions with respect to the choice of template and cross-linking monomer proved critical and resulted in a polymer which strongly and selectively bound riboflavin in water-rich media similar to those found in common alcoholic beverages.[8]

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For the design of the polymers, we first turned our attention to the functional monomer. The flavin ring system contains an imide functionality which contains an acceptor-donor-acceptor (ADA) array of hydrogen-bonding sites capable of interacting with receptors containing a complimentary DAD array. Representative of such molecules are 2,6-bisamidopyridines, whose ability to bind to imide functionalities has been widely studied.[9] Accordingly, we chose 2,6-bis(acrylamido)pyridine (BAAPy) as our functional monomer (Scheme 1).[10,11]

With regards the choice of template, we focused on flavin and riboflavin derivatives having high solubility in typical imprinting solvents, in this case chloroform (Scheme 1), initially attempting to use N(10)-alkylflavins.[12] However, the solubility of these compounds in chloroform was found to be too low (ca. 16 mM for **1** and only ca. 6 mM for **2**) for a conventional template/monomer molar ratio to be used. As expected, polymers prepared using these analogues as templates exhibited low imprinting factors and capacities (see below). Riboflavin tetraesters performed better in this regard, their solubility in chloroform being far greater than that of the N(10)-alkylflavins described above (RfAc: 0.2 M, RfPr: 0.6 M, RfBu: 0.8 M). Furthermore, the use of these tetraesters of various sizes allowed the investigation of other effects of the templating process, for example, size-exclusion phenomena. A ^{1}H NMR titration was performed in $CDCl_3$ using **1** as the guest and BAAPy as the host to check the likely effects of these pairings of template(s) and functional monomer. The raw data were fitted by nonlinear regression to a 1:1 binding isotherm and an association constant of $K_a = 570 \pm 35\ M^{-1}$ was extracted.[13] This value is in accordance with association constants obtained previously with similar systems,[14] and implies that the major part of the template is complexed prior to polymerization. The magnitude of the K_a value, along with the observed complexation-induced shifts of protons in the NMR spectra of both the host and guest, support the presence of a three-point hydrogen-bonded complex as depicted in Figure 1.

The use of the riboflavin esters as templates leads to MIPs[15] that exhibit extremely strong retention of their respective templates (Figure 2). Indeed, modification of the mobile phase with acetic acid was necessary to reduce the retentions to a workable time frame. Anticipating that the primary electrostatic driving force causing this retention was the three-point hydrogen-bond array, we also challenged the RfAc-imprinted and control polymers with the *N*-methylated template analogue **6**. We observed total suppression of the retention on both the imprinted and control polymers, thus providing proof that the complexation mode observed in solution is also preserved in the polymer. Furthermore, evidence of size-exclusion effects is seen in the different polymers. Thus, P(RfAc) retains its template to a greater extent than the other analytes, while the retentions of RfAc and RfPr on P(RfPr) are very similar. This result suggests that the polymeric binding site formed around RfAc is able to exclude the larger analytes, while the polymeric binding sites formed around RfPr appear to still offer a "good fit" for the smaller analyte. The larger analyte RfBu is less strongly retained on both these polymers. However, the retention of

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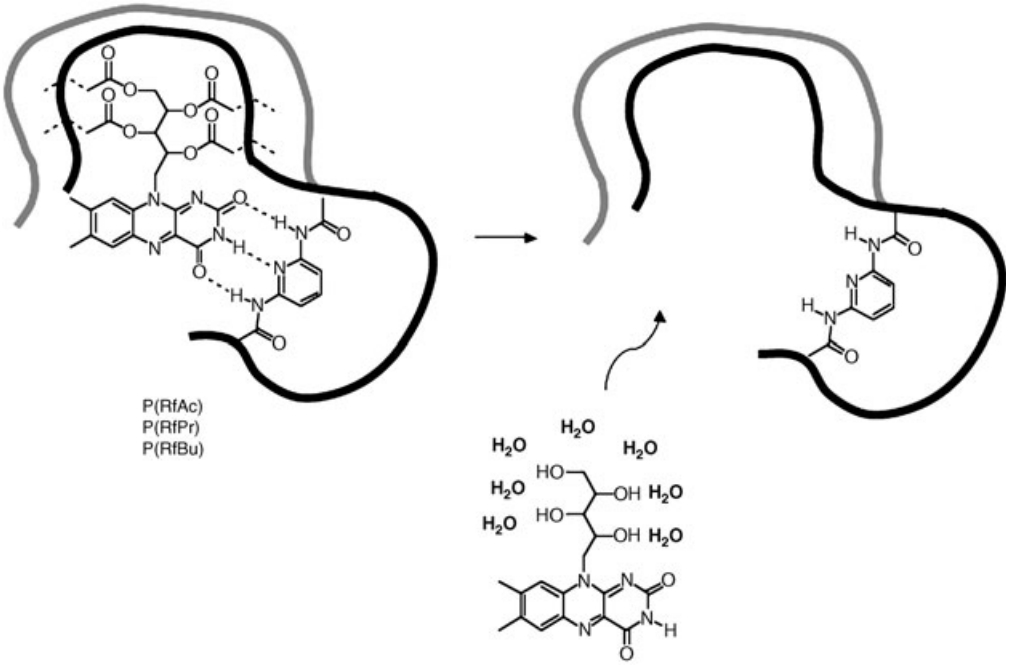
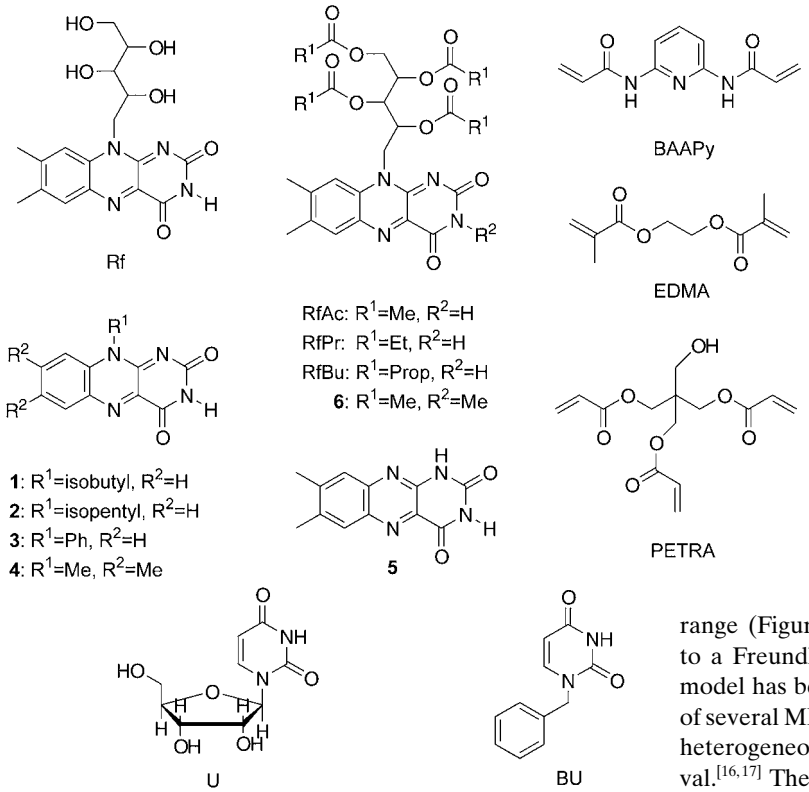


Figure 1. Fine-tuning of the size of a binding cavity complementary to the ribose chain of riboflavin (Rf) using riboflavin tetraesters as templates.



Scheme 1. The monomersand templates investigated in the study.

RfBu is strongest on P(RfBu), perhaps indicating that, while the binding sites formed in these polymers are large enough to accommodate RfAc and RfPr, there is a lessening of secondary interactions provided by the polymeric matrix, as the templated cavity is now too large for these smaller analytes. This view is further supported by the much lower retentions of the other smaller analytes (BU and **3**–**5**) on all polymers. The large imprinting factors arise from two effects, namely the pronounced retention of the riboflavin analytes on the MIPs coupled with low retention on the control, non-imprinted polymer P(N). This observation suggests that the binding sites provided by the functional monomer units in P(N) are located in areas of the polymeric matrix that cannot be accessed by the larger tetraester analytes. This assumption is again supported by the comparatively stronger retentions of the smaller analogue analytes on P(N).

Insights into the affinities and abundance of these sites were obtained by frontal analysis (low concentration range, 0–1 mM) and batch partitioning experiments (higher concentration range, 0–20 mM). In the low concentration range (Figure 3 a) the isotherms could be excellently fitted to a Freundlich multiple site model (Figure 3 a inset). This model has been shown to describe well the binding behavior of several MIPs in the low concentration range and indicates a heterogeneous distribution of binding sites within this interval.[16,17] The isotherms obtained in the higher concentration interval (Figure 3 b) display a sigmoidal-type saturation behavior, the origin of which is currently unclear.

As suggested from the earlier chromatographic results, binding to the non-imprinted polymer is confirmed to be weak. The isotherms of the imprinted polymers also agree with the relative retentions observed in the chromatographic investigation. Thus, P(RfAc) shows the highest binding capacity, exceeding that of P(N) by more than 30 μmol g^{-1}

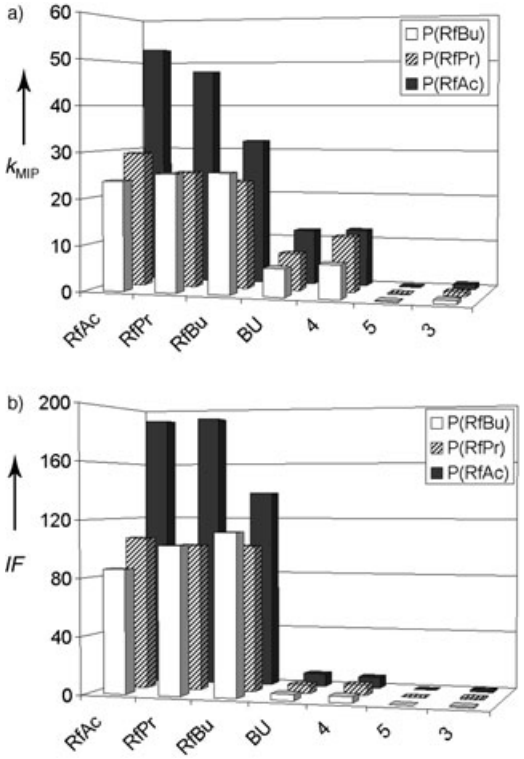


Figure 2. a) Retention factors (k) and b) imprinting factors (IF) obtained in the chromatographic evaluation of the riboflavin tetraester imprinted polymers (P(RfAc), P(RfPr), P(RfBu)) and a control, non-imprinted, polymer (P(N)) using acetonitrile/acetic acid (99/1 v/v) as the mobile phase. k and IF are defined as follows: $k=(t-t_0)/t_0$; $IF=k_{MIP}/k_{NIP}$; where t = retention time of the solute; t_0 = retention time of a nonretained void marker; $k_{MIP}(k_{NIP})$ = retention factors for a given solute using the imprinted (or non-imprinted) polymer as stationary phase.

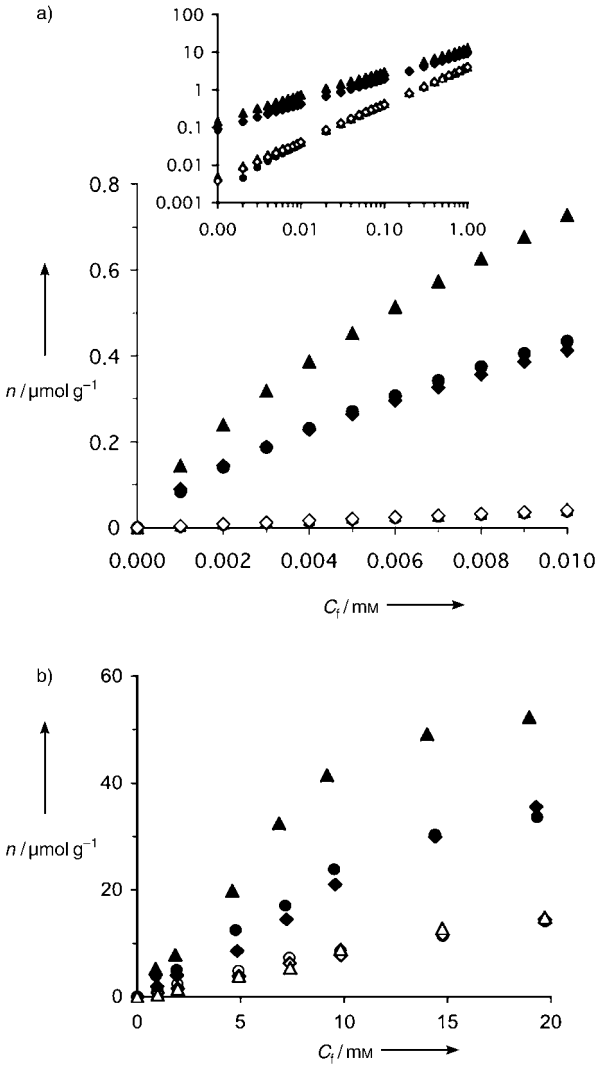


Figure 3. Binding isotherms of riboflavin tetraesters (RfAc: triangles, RfPr: circles, RfBu: diamonds) on their respective complementary polymers (P(RfAc), P(RfPr), P(RfBu): filled symbols) and on a control non-imprinted polymer (P(N); open symbols) as determined a) by frontal analysis using the staircase method or b) by a batch partitioning experiment in acetonitrile/acetic acid (99/1 v/v). In (b) the polymers (10 mg) were equilibrated for 24 h with solutions (0.5 mL) containing different concentrations of the riboflavin tetraesters.

at saturation. The binding curves of P(RfPr) and P(RfBu) exhibit similar shapes, but with lower binding of the templates. Thus, the reason for the stronger retention of RfAc on its complementary polymer appears to be a consequence of a larger number of sites rather than the presence of sites of higher affinity. Nevertheless, high-energy binding sites are clearly present. Thus, at a free concentration (C_f) of 1 µM, P(RfAc) adsorbed 0.15 µmol RfAc g^{-1} polymer with negligible nonspecific adsorption, thus indicating the presence of sites with $K_a > 10^6\,M^{-1}$.

The primary goal of our study was the recognition of riboflavin in aqueous-based media. To this end, the above polymers were tested, again in the chromatographic mode, for their ability to retain Rf under such conditions (Figure 4).

Retention of Rf by **P1** is weak and only minimally different from the retention of Rf by the control P(N). Thus, although **P1** is able to recognize its template over analogous molecules in organic media, **1** is a poor template for the preparation of MIPs that exhibit Rf recognition in aqueous media. For the MIPs prepared using the riboflavin esters as templates, much stronger retentions of Rf are observed, with P(RfAc), P(RfPr), and P(RfBu) all capable of retaining Rf to a much greater extent than either P(N) or **P1**. The relative retention of Rf on these polymers agrees with their relative binding capacities for their own templates (Figure 3). Thus, P(RfAc) emerges as the MIP best able to retain Rf under the conditions tested, which implies that the binding sites formed around this tetraester offer a good fit for the ribose chain of Rf as well.

These encouraging results were somewhat offset by the performance of the polymers in pure water or water containing small amounts of additives. In this case, nonspecific, hydrophobically driven binding dominated the retention, thus leading to much lower imprinting factors. To suppress this contribution, the polar cross-linking monomer pentaerythritol triacrylate (PETRA) was used in place of EDMA. The corresponding polymer (P(RfAc)′) retained Rf strongly, with a retention almost two times higher than P(RfAc). Furthermore, the retention displayed by the control polymer (P(N)′) was lower than that of the corresponding EDMA-based

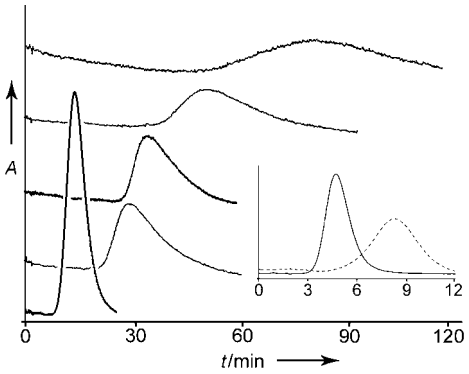


Figure 4. Elution profiles of riboflavin (10 µl of a 1 mM solution) injected on columns packed with from bottom to top (main figure): P**1**, P(RfBu), P(RfPr), P(RfAc), and P(RfAc)′. The inset shows the elution profiles of riboflavin on columns packed with P(N) (dashed line) and P(N)′ (solid line). Mobile phase = water/acetonitrile/ethanol (85.5/10/4.5 v/v/v).

control P(N) (see inset in Figure 4). Thus, it appears that the hydroxy groups of the cross-linking monomer create both a more hydrophilic backbone with lower affinity for nonpolar compounds and provide additional stabilizing interactions in the imprinted sites, presumably through hydrogen bonds to the ester function of RfAc. Frontal analysis on this polymer confirmed these findings. Thus P(RfAc)′ bound roughly two times more template at a given solution concentration than P(RfAc) and displayed more uniform high-affinity sites.

P(RfAc)′ was then subjected to a thermodynamic investigation using isothermal titration microcalorimetry (ITC). This technique has proven extremely versatile for the thermodynamic characterization of receptor–ligand interactions,[18] including an imprinted system,[19] and is based on the measurement of the heat changes occurring upon titration of a receptor (or a ligand) with its binding partner. The addition of a dilute solution of Rf to a suspension of the polymer particles ($0.9\,mg\,mL^{-1}$) in a beverage-mimicking solution (water/formic acid/ethanol: 90.6/4.7/4.7 (v/v/v)) led to exothermic heat pulses, thus indicating noncovalent interactions between the titrant and the suspended polymer particles. The heat generated per addition was calculated by integrating the heat pulses and thereafter plotted against the total concentration of Rf in the cell (Figure 5 a). Within this small concentration interval, limited by the analyte solubility, the heat generated per addition was constant, with no evidence of saturation. Nevertheless, clear differences between the polymers were observed. Interaction of Rf with the imprinted polymer was exothermic, exceeding that with the nonimprinted polymer by more than $1.3\,kcal\,mol^{-1}$, with the latter signal coinciding with the background signal obtained by addition of riboflavin in the absence of polymer.

The presence of highly discriminating imprinted sites was further supported by the absence of any such effect when the control analyte uridine was added. Uridine, which contains both the imide and ribose substructures, could be expected to cross-react with sites designed to bind riboflavin. However, the signal observed when this analyte was added to the different polymers did not differ from the blank runs.

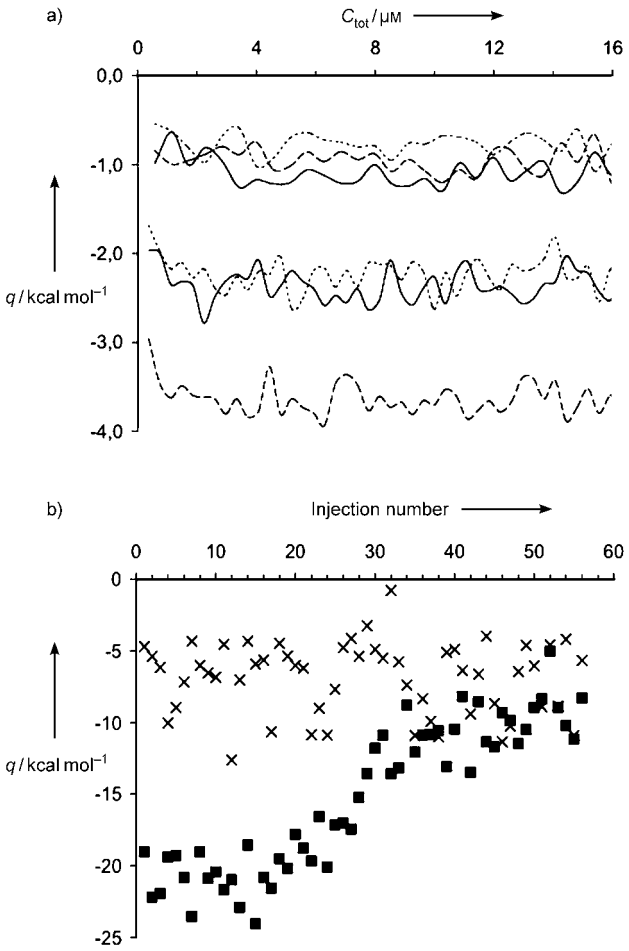


Figure 5. ITC titration profiles showing in a) the energy released (q) versus the total concentration of the titrant in the cell (C_{tot}) for the titration of P(RfAc)′ (dashed lines), P(N)′ (solid lines), or blank (dotted lines) with Rf (lower three profiles) or U (upper three profiles) in water/ethanol/formic acid: 90.6/4.7/4.7 (v/v/v), and b) the energy released versus the injection number for the titration of solutions of Rf (■) or U (X) (1 µM in 50 mM aqueous sodium phosphate buffer pH 7) by RfBP.

Unfortunately, the limited solubility of riboflavin meant that only a fraction of the binding sites could be investigated in these experiments. Thus, none of the thermodynamic quantities typically furnished by this type of experiment could be estimated. However, the presence of a specific exotherm at a low total concentration of analyte (<400 nM) implies that binding sites with affinities $>10^6\,M^{-1}$ are present. The absence of saturation effects further indicates that the sites within this population are relatively uniform.

Finally, we compared the imprinted receptor with its biological counterpart—riboflavin binding protein (RfBP). This protein was titrated by the classical technique in two different solvent systems. Pronounced exothermic heat pulses were observed under neutral conditions, with saturation clearly occurring close to a receptor:ligand stoichiometry of 1:1. Analysis of the saturation curve provided a binding constant of $9.0(\pm0.5)\times10^5\,M^{-1}$ (four independent measurements), with an associated enthalpy of $9.4(\pm0.1)\,kcal\,mol^{-1}$. Similar to the situation with the imprinted receptor, no signal

was observed when the control analyte uridine was used as the titrant. A different picture emerged when the protein was assessed in the beverage-mimicking solution used to evaluate the imprinted polymer. A pronounced exotherm was observed under these conditions upon each addition, with no apparent binding-related signals, which implies that the protein denatures under conditions where the imprinted polymer retains its ability to bind the target. Notably, this occurs in a solvent system which resembles that found in common alcoholic beverages.[8]

We are currenttly investigating means to further enhance the affinity of the polymers for riboflavin and to suppress the nonspecific binding occurring in pure water.

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