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Tetrahedron Letters 46 (2005) 5651–5654
Tetrahedron Letters

First example of a molecularly imprinted polymer incorporating a difunctionalized alloxazine flavin isomer

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Received 11 May 2005; revised 18 June 2005; accepted 20 June 2005
Available online 7 July 2005

Abstract—High-quality alloxazine (a flavin isomer) imprinted polymers have been made for the first time. A molecularly imprinted polymer (MIP) for the N1,N3-di-functionalized alloxazine template **2** was made. The MIP prepared for **2** exhibited excellent, highly selective molecular recognition for template **2**, as determined by HPLC analysis using columns prepared with the MIP. This has also demonstrated that the core flavin structure can survive the imprinting process.

Molecularly imprinted polymers have been widely applied in chromatography[1] and analytical chemistry.[2] Recently efforts have been made to extend their applications into fields such as sensors[3] and catalysis.[4] Imprinting with a molecule that is non-covalently bound to the polymer creates sites of molecular recognition within the polymer structure that are based on the shape of the imprinting molecule as well as the characteristics of the functional group interactions between the template and the monomers, such as ionic bonding, H-bonding, dipolar bonding, and Van Der Waals interactions.

Flavins are involved in a large number of biological processes and exist in living things in the forms, flavin adenine dinucleotide (FAD) and flavin mononucleotide (FMN).[5] These are typically redox cofactors involved in 1 and 2 electron redox reactions in enzymatic reactions.[5,6] In order for a flavin to be able to efficiently perform redox reactions it is necessary for the flavin to be in catalytic active sites in enzymes.[7] Our long-term goal is to develop flavin based redox catalysts using flavin-imprinted molecularly imprinted polymers as mimics of flavin-utilizing enzyme redox catalysts.

In order to create an imprinted site, the templating molecule (flavin) must be able to coordinate to a molecule that will be involved in the polymerization and thus be incorporated into the polymer. In this system, we chose to take advantage of the interaction of carboxylic acids with amine bases in functional monomers, using pyridine groups in 4-vinylpyridine monomers.

The synthesis of the imprinting alloxazine template **2** (Scheme 2) was achieved in two steps from lumichrome.[8] The dialkylation of lumichrome with base used the same procedure for both **3** and **4**.[8] The dialkylation reaction of lumichrome yields an alloxazine, which is a non-redox active isomer of flavins (isoalloxazines).[9]

Imprinted polymers have been shown to be most effective when using a high level of crosslinking monomer.[10] The imprinted polymer was synthesized using ethylene glycol dimethacrylate (EDMA) and methyl methacrylate (MMA). Radical polymerizations were done using V-70 (2,2′-azobis(2,4-dimethyl-4-methoxy)valeronitrile), a radical initiator that can form radicals at room temperature at a practically useful rate.[11] This has advantages over the more commonly used 2,2′-azobis(2-methylpropionitrile) (AIBN), which requires higher temperatures to form radicals. Using V-70 the polymerization can occur just above room temperature, which is advantageous since lower temperatures can improve the quality of imprinted sites in MIPs.[12] The polymerization was carried out in air-free, dry THF under nitrogen. An imprinted polymer was made using **2** as the template complexed in solution with 4-vinylpyridine, using 90% EDMA crosslinker, as depicted in Scheme 1.

Keywords: Molecularly imprinted polymer; Flavin; Alloxazine; Enzyme mimetic; Redox catalyst.
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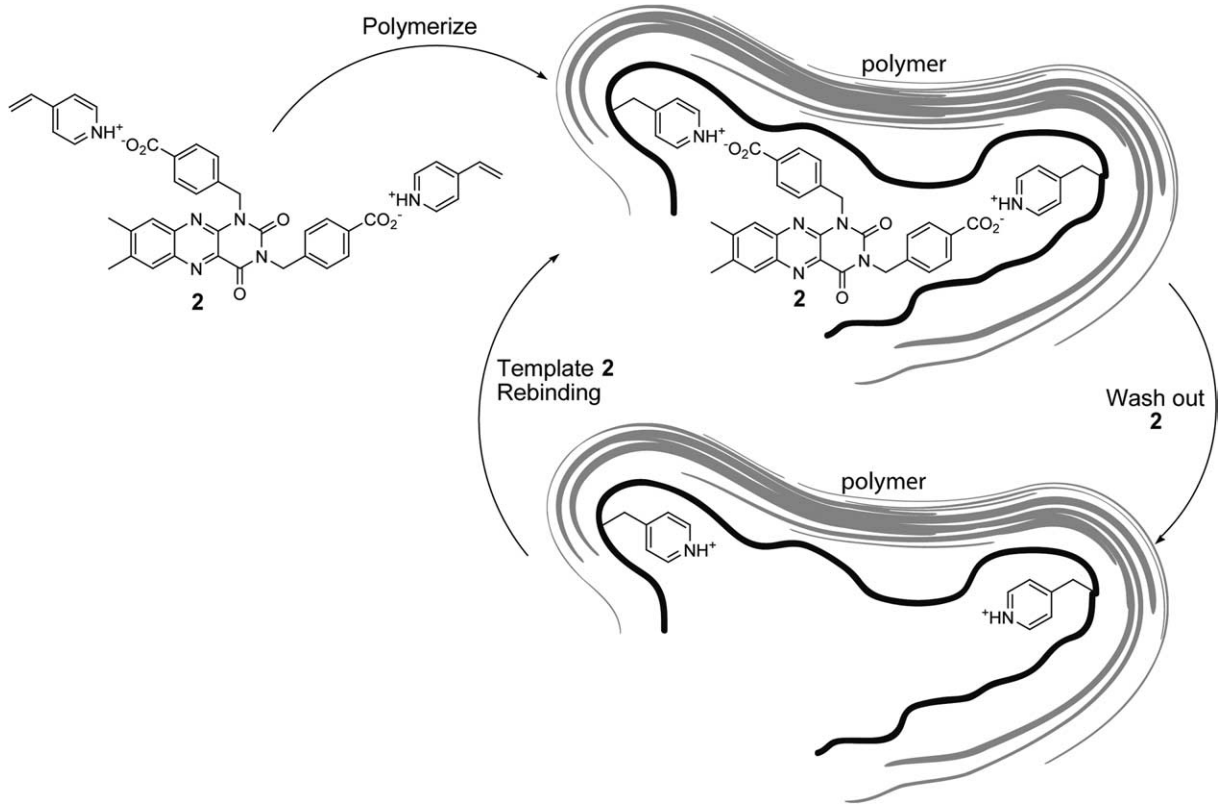
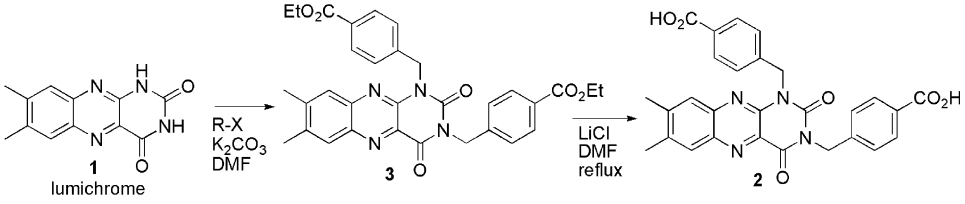
doi:10.1016/j.tetlet.2005.06.102



Scheme 1. Formation of a molecular recognition site within the polymer.



Scheme 2. Reagents and conditions: (a) DMF, K_2CO_3, ethyl 4-(bromomethyl)benzoate, 90 °C, 16 h; (b) LiCl, DMF, reflux 3 days.

Although alloxazine **2** and related alloxazines **3** and **4** are not true flavins, the disubstituted alloxazines are easy to prepare and conveniently provide a system to examine the quality of the imprinted sites using a flavin-like difunctionalized template, alloxazine **2**.

A good imprinted polymer should show high selectivity toward rebinding the molecule used as a template. One of the best and most common methods for testing the molecular recognition of an imprinted polymer is through its use as a stationary phase in HPLC.[13] The template should be retained on the column significantly longer than other molecules with different molecular shape and/or binding characteristics. A simple way to measure this is through the calculation of the capacity factor k,[14] calculated as $k = (t_g - t_o)/t_o$ where t_o is the retention time of the void marker and t_g is the retention time of the guest to be bound. A good k value should be significantly larger (>1) for the template compared to the template analogs. The selectivity factor (α)[15] is a way of directly comparing polymer selectivity between template analogs and is calculated using $\alpha = (k_{analog}/k_{template})$. In order to determine whether or not the imprinted polymer retained memory for the template, a series of analogs were used to compare with the binding characteristics of the template (**2**).

HPLC results: An empty HPLC column was packed[16] with the imprinted polymer of **2** and was subsequently used to measure the binding of **2**–**6**. The retention times, k, and α for **2**–**6** were measured and are given in Table 1.

Table 1. Guest binding of template and analogs for the imprinted polymer as HPLC stationary phase

Guest	Retention time (min)	Capacity factor k	Separation factor α
2	14.7	4.26	1
3	9.31	2.33	0.55
4	9.63	2.44	0.57
5	3.45	0.23	0.05
6	8.5	2.04	0.48

t_o = 2.8 min (acetone) used in the calculation of k.

All reported data were measured from the same column packing by using identical and isocratic solvent conditions.

The template **2** had the longest retention time out of all the compounds tested. This is to be expected because the binding sites created in a MIP are formed around the template and thus should be most complementary to the template. The ionic and H-bonding interaction between carboxyl groups on **2** and 4-vinylpyridine monomers are locked into place in a specific geometry when the 4-vinylpyridine is polymerized. This creates sites within the polymer matrix, which have a memory for the template. The MMA and EDMA monomers are also capable of H-bonding to the template, and create a 'pocket' inside the polymer based on the specific shape of the template and the H-bonding interactions with the flavin core. The k value for **2** represents a template, which is strongly bound to the MIP. A recent example using MIPs imprinted with cholesterol[17] cited k values between 3.1 and 4.0 as evidence for excellent molecular imprinting of high-quality binding sites in the MIPs. This is a typical example. Compounds **3** and **4** are direct analogs of the template **2**, where the key difference is the lack of the carboxylic acid. The k and α values for each are similar, and while they show significant binding it is considerably less than the template **2**. Because there is no ionic interaction with **3** and **4** and the MIP, their recognition can only be based on their flavin core structure. This can be explained as polar bonding interactions between polar groups in the polymer in the imprinted binding site and polar groups and rings in the flavin core. Riboflavin **5** was chosen as a guest compound to study because it has the core flavin ring system but is not as structurally similar to the template as **3** and **4**. It is evident by the results with **5** that it is poorly recognized by the MIP. The structure of riboflavin **5** is too different from **2** to **4** to be bound effectively within the imprinting site. This demonstrates that the imprinted polymer has specific sites of molecular recognition based on the templating structure. Benzoic acid **6** was chosen as a simple acid to test the binding interactions with pyridine groups incorporated into the MIP. It is very small compared to the template and should be bound to the MIP based solely on ionic and H-bonding interactions between the carboxylic acid and the pyridine base. The k and α values for benzoic acid show significant binding to the MIP. The binding ability of **6** represents the strength of a carboxyl-pyridine interaction as the MIP acts like an ion-exchange polymer. Not only will **6** have interactions within the imprinted sites, but also there are potential interactions with pyridines randomly incorporated in the MIP as a consequence of the 8-fold molar excess of functional monomer used in these studies to ensure complete complexation of template 2 by 4-vinylpyridine monomers. The binding studies of compounds **2**–**6** demonstrate the strong and selective molecular recognition of the flavin imprinted site (Fig. 1).

As a control, a column was packed with a polymer that was formed with identical reactants and conditions except that template **2** was not included. This should lead to no molecular imprinting effect. An HPLC column was prepared for the non-imprinted polymer in the same way that the HPLC column was prepared for the imprinted polymer above. HPLC studies of the imprinting template **2** with the non-imprinted polymer control gave a retention time of 2.7 min, which was nearly the same as the acetone void marker for the control column (2.6). This demonstrates the importance of molecular imprinting in molecular recognition in this system.

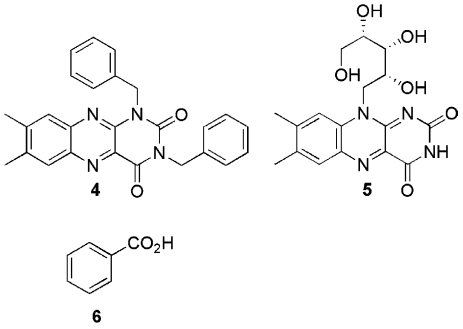


Figure 1. Additional molecules used to study molecular recognition by the imprinted polymer.

In conclusion, these studies demonstrate two key points. First, the studies show that template **2** survives the standard free radical polymerization process to produce molecularly imprinted polymers. Second, the HPLC studies using a molecularly imprinted polymer prepared with a highly functionalized flavin-like alloxazine **2** demonstrates that high-quality and specific sites of molecular recognition can be produced using a flavin-like template. In future work, the results of these studies will be used to guide the synthesis of polyfunctionalized, catalytically competent flavin templates to produce mole- cularly imprinted polymers with highly specific, catalytically active imprinted sites for flavin-utilizing enzyme-mimetic redox catalysis.

Acknowledgements

This research has been supported by the University of Oregon Department of Chemistry and Materials Science Institute.

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