A molecularly imprinted polymer receptor for the enantiomeric recognition of amino acid hydantoins mimicking cooperative hydrogen bonds between nucleotide bases

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Using acrylamide as hydrogen bonding functional monomer and (5*R*)-5-benzylhydantoin as template, a molecularly imprinted polymer was prepared in a polar solvent, which exhibited good enantiomeric recognition properties. The binding characteristics and selectivity of the polymer were evaluated by batch methods. Scatchard analysis showed that two classes of binding sites were produced in the polymer matrix and their dissociation constants were calculated to be 3.5×10^{-5} mol/L and 4.3×10^{-4} mol/L, respectively, by utilizing the model of multiple independent classes of binding sites. These results were more reasonable than those obtained by Scatchard analysis, which was in agreement with the prediction of the binding characteristics of the polymer by exploring the effect of acrylamide on UV spectra of (5*R*)-5-benzylhydantoin. The substrate- and enantio-selectivity of the polymer was investigated. Finally, the study of effect of water on the chiral separation factor of the polymer further proved that the hydrogen bonding interactions played an important role in the recognition of the acrylamide-based molecularly imprinted polymers.

Keywords (5*R*)-5-Benzylhydantoin, molecular imprinting, molecular recognition, multisite binding model, enantioselectivity

Introduction

Considerable efforts have been made to create recognition materials with binding properties that resemble those of natural binding entities such as antibodies and receptors.[1] Molecular imprinting has received much attention to produce the materials in recent years because it allows the formation of specific recognition and catalytic sites in polymer matrix without elaborating molecular designs and multi-step synthesis.[2-5] The selected ligand or template is first allowed to interact via covalent and/or non-covalent bond formation with one or more functional monomers freely in solution to form host-guest complexes. The resulting host-guest complexes are subsequently co-polymerized with a large excess of crosslinker to give a rigid insoluble polymer. After extraction of the template, specific recognition sites are left in the polymer network where the spatial arrangement of the complementary functional groups of the polymer matrix, together with the shape image, corresponds to the template.

Over the years, the molecularly imprinted polymers have been used for a wide range of applications ranging from stationary phase for chiral HPLC separations[5,6] to the enrichment/concentration of samples in biomedical, environmental and food analysis.[7-10] In previous studies, the most widely used functional monomers were methacrylic acid (MAA). It could be assumed to interact via ionic interactions with amines and via hydrogen bonds with amides, carbonates and carboxyls. However, some studies[11,12] have shown that when only hydrogen bonding interactions between the template molecule and the carboxyl monomers are involved, the hydrogen bonding interactions are very weak in polar solvents and the MIPs made in polar solvents exhibit only very weak enantiomeric recognition and in some case no recognition at all. Amino acid hydantoins are available to the stereochemistry of amino acids and can be applicable for the

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amino terminus's determination of peptide,[13] nevertheless, most of them are insoluble in non-polar solvents and have weak acidity. It can be expected that it is difficult to use MAA as a functional monomer in molecular imprinting. Here, using acrylamide as functional monomer, a (5*R*)-5-benzylhydantoin-imprinted polymer was synthesized in acetonitrile mimicking cooperative hydrogen bonds between nucleotide bases in bio-affinity processes. This study indicated that the acrylamide-based polymer exhibited good substrate- and enantio-selectivity for the original template molecules (5*R*)-5-benzylhydantoin. It is possible to further enlarge the application of molecularly imprinted polymers to the development of a new method for effectively recognizing peptide and protein.

Experimental

Materials and instruments

L- and *D*-phenylalanine were purchased from E. Merk. Potassium cyanate was from Fluk. 5,5-Diphenylhydantoin (DPH) was from Aldrich. Acrylamide, iodomethane, dimethyl sulphate, methanol, acetic acid and acetonitrile were from Tianjin No. 2 Chemical Reagent Factory. 2, 2′-Azobiisobutyronitrile (AIBN) was from the Special Reagent Factory of Nankai University. Ethylene glycol dimethacrylate (EDMA) was prepared from ethylene glycol and mathacrylate acid. Acetonitrile was of chromatographic grade and other chemicals were of analytical grade.

A Shimadsu UV-240 double-beam spectrophotometer, an FT-NMR Model FX-90 (JEOL) and a Model SHZ-82 constant temperature bath oscillator were used.

Synthesis of methyl derivatives of DPH

3-Methyl-5, 5-diphenylhydantoin (MDPH) 2 mL of iodomethane was dropped under magnetic stirring into a solution of 2.50 g of DPH in 100 mL of 0.011 mol/L NaOH. The reaction was allowed to proceed for 3 h at 25℃ and the white precipitate was collected by filtration and recrystallized from EtOH. Yield: 67%, mp: 215—216℃ (Lit. 217℃), ^{1}H NMR ($CDCl_3$): 3.07 (N3-CH_3), 7.40(ArH), 8.21(N1-H).

1, 3-Dimethyl-5, 5-diphenylhydantoin (DMDHP) 50 mL of dimethyl sulphate was added dropwise under magnetic stirring into a solution of 1.25 g of DPH in 250 mL of 2 mol/L NaOH and was prepared according to the procedure of the preparation of MDPH. Yield: 73%, mp: 196—197℃ (Lit 197℃), ^{1}H NMR (CD-Cl_3): 2.80(N1-CH_3), 3.12(N3-CH_3), 7.38(ArH)

Preparation of template and its enantiomer

(5*R*)- and (5*S*)-5-benzylhydantoin (*R*-BZH and *S*-BZH) were prepared as described in the literature.[14] *L*- or *D*-phenylalanine (1.652 g, 10 mmol) and potassium cyanate (0.9735 g, 12 mmol) were dissolved in water (10 mL) and were allowed to react for 30 min at 70℃ under stirring. The reaction mixture was refluxed with 6 mol/L hydrochloric acid (10 mL) for 2 h, then slowly cooled to room temperature, then the crystalline products were produced. Recrystallization from hot water gave 1.50 g of *R*-BZH or 1.56 g of *S*-BZH in 79.4% or 82.0% yield. *R*-BZH: mp 179—181℃, $[\alpha]_D$ −181°(ethanol); ^{1}H NMR (DMSO-d_6): 3.01(CH_2), 4.23(C5-H), 7.28(ArH), 7.76(N1-H), 10.37(N3-H). *S*-BZH: mp 179—180.5℃, $[\alpha]_D$ + 181°; ^{1}H NMR spectrum was as the same as that of *R*-BZH.

R-BZH-Imprinted polymer (P_{RBZH}) *preparations*

1 mmol of the template *R*-BZH was dissolved in acetonitrile (10 mL) in a 30 mL glass ampoule. To the solution were added 4 mmol of acrylamide (functional monomer), 20 mmol of EDMA (cross-linking monomer) and 50 mg of AIBN (initiator). After nitrogen gas sparged into the solution for 5 min, the ampoule was sealed under vacuum, and kept in a shaker bath at 60℃ for 24 h. The resultant bulk rigid polymer was ground to pass through 75-μm sieve. Fine particles were removed by repeated sedimentation in acetone. The resulting particles were placed into a home-made extraction apparatus[15] and fully washed at 1.0 mL/min of the flowrate under continuous stirring condition with 1:9 (*V*/*V*) acetic acid to methanol, then washed with acetonitrile again until the *R*-BZH could no longer be detected at 205 nm in the eluent. Finally, the resulting particles were dried to constant weight under vacuum at 60℃. A reference non-imprinted polymer (P_0) was prepared in the same way without the addition of *R*-BZH.

Binding experiments

The sized and washed polymer particles (20.0 mg)

were placed in a 10 mL conical flask and mixed with 2.0 mL of a known concentration of *R*-BZH or other substrate acetonitrile solution. The conical flask was oscillated in a constant temperature bath oscillator at 25℃ for 12 h. The mixture was transferred into a centrifuge tube and centrifuged for 5 min. The concentration of free *R*-BZH or other substrates in the solutions was determined using a spectrophotometer at appropriate wavelength. The amount of substrates bound to the polymer Q was calculated by subtracting the concentration of free substrates from the initial substrate concentration. The average data of triplicate independent results were used for the following discussion.

Spectrophotometric analysis

A series of solutions were prepared with a fixed concentration of *R*-BZH (1.0 mmol/L) and varied amount of acrylamide or MAA in acetonitrile. A_{max} and λ_{max} of the absorption spectra of these solutions were determined with corresponding acrylamide or MAA solution as references.

Results and discussion

Prediction of the binding properties and selectivity of P_{RBZH}

The studies of the rational design for the preparation of P_{RBZH} and the recognition mechanism of the polymer would be important to understand the imprinting and recognition phenomena. By the theoretical analysis of the interactions of *R*-BZH with MAA or acrylamide and the study of effect of MAA and acrylamide on UV spectra of *R*-BZH, the rational design for the preparation and the binding selectivity of P_{RBZH} may be predicted. Theoretically, amide group may form stronger hydrogen-bonding interactions than carboxylic group in polar solvents. This reason can be explained as follows: (i) The dielectric constant and dipole moment of the amide group are much higher than those of the carboxylic group. For example, acetic acid has a dielectric constant of 6.20 and a dipole moment of 1.70 D, while for acetamide these values are 67.6 and 3.76 D.[16](ii) In a peptide, the amide oxygen has 0.42 negative charge and the hydrogen 0.20 positive charge.[17] The same conclusion can also be drawn by comparing the effect of MAA and acrylamide on the UV spectra of *R*-BZH. At a fixed concentration of *R*-BZH and different concentrations of MAA or acrylamide, two series of solutions were prepared in acetonitrile. The effect of MAA and acrylamide concentration on A_{max} and λ_{max} of *R*-BZH was given as shown in Fig. 1, which showed that when the concentrations of acrylamide or MAA increased from 0.0 to 6.0 mM at 1.0 mM of *R*-BZH the maximum absorption wavelengths λ_{max} of *R*-BZH shifted from 205 nm to 248 nm or 228 nm, with corresponding pure monomer solutions as references. The bigger effect of acrylamide on λ_{max} of *R*-BZH than MAA indicated that acrylamide can induce stronger interaction with *R*-BZH than MAA. Fig. 1 also showed that the maximum absorbances A_{max} at λ_{max} decreased with the addition of acrylamide or MAA. However, acrylamide made the reduction more rapid as compared with MAA, for example, when the concentration acrylamide or MAA is 0.5 mM, A_{max} is decreased to 0.585 or 1.512. It is of interest to note that A_{max} of *R*-BZH tends to become constant as the concentration of acrylamide is no less than 4.0 mM. Under the same conditions, A_{max} of *R*-BZH still obviously reduces with MAA instead of acrylamide. The fact further indicates that acrylamide can form more stable cooperative hydrogen-bonded complexes with *R*-BZH than MAA. It is easier to make the more

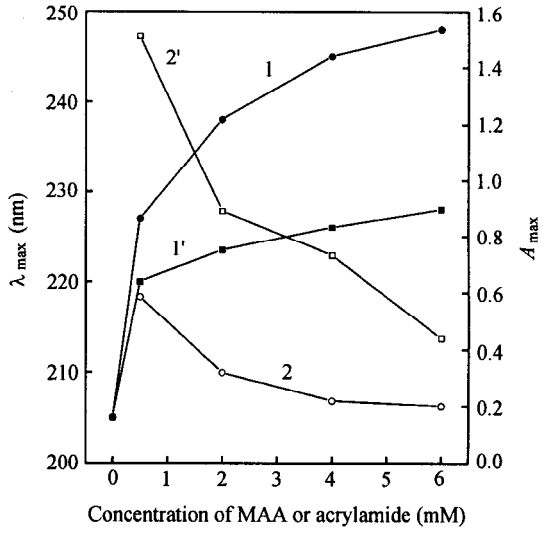


Fig. 1 Effect of acrylamide or MAA on λ_{max} and A_{max} of *R*-BZH in acetonitile. Curves: 1, λ_{max} vs. acrylamide; 2, A_{max} vs. acrylamide; 1′, λ_{max} vs. MAA; 2′, A_{max} vs. MAA; $L = 1$ cm; corresponding pure acrylamide or MAA solutions as references.

stable hydrogen-bonded host-guest structure be preserved into the polymer matrix by crosslinking in the presence of a large excess of crosslinker. So the acrylamide-based molecularly imprinted polymer prepared in polar solvents can exhibit higher selectivity for the original template molecules than the MAA-based polymer.

According to the molecular structures of *R*-BZH and acrylamide and the changes of A_{max} and λ_{max} of *R*-BZH with addition of acrylamide as those shown in Fig. 1, the structural models of the hydrogen-bonded complexes formed between acrylamide and *R*-BZH could be the same as those shown in Fig.2.

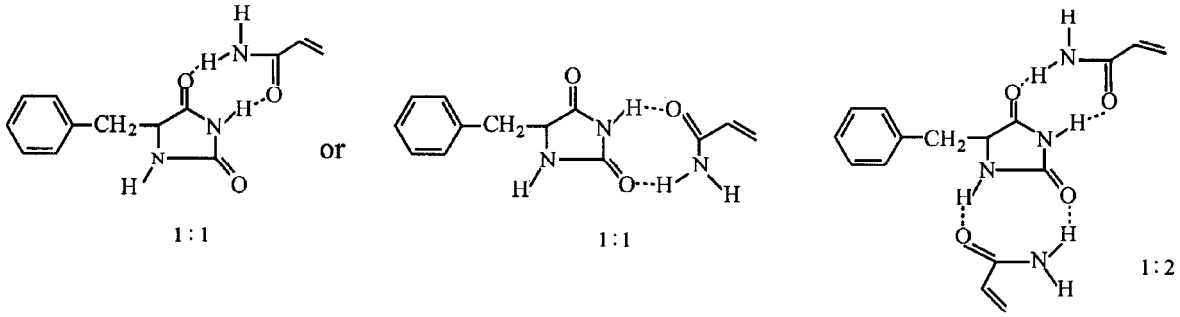


Fig. 2 Possible structures of *R*-BZH-acrylamide hydrogen-bonded complexes.

In addition, some conclusions can be speculated on from the effect of acrylamide on A_{max} and λ_{max} of *R*-BZH as shown in Fig. 1. Firstly, the formation of 1:1 complexes rather than 1:2 complexes should be predominant when the template *R*-BZH is present at higher template to monomer ratio. From a practical standpoint, this situation mainly presents low selective binding sites in the imprinted polymer P_{RBZH}. Secondly, the higher the initial concentration of acrylamide, the higher concentration of the 1:2 complexes could be present in the mixture. The situation can result in high selective binding sites in P_{RBZH}. Clearly, the increase of initial concentration of acrylamide is rather favourable for enhancing the number of high selective binding sites in P_{RBZH}. However, a large excess of acrylamide can also lead to obviously increasing the non-selective binding sites induced by non-assembly functional monomer acrylamide. In fact, the selectivity of P_{RBZH} can decrease. Taking the above mentioned aspects into account, we selected acrylamide as functional monomer and the template/monomer molar ratio as 1:4 to prepare the imprinted polymer P_{RBZH}. Afterwards, its binding characteristics and selectivity were explored.

Binding characteristics of P_{RBZH}

In the binding study of MIPs, it has been found that two classes of binding sites often existed. The binding parameters of MIPs were mostly estimated by Scatchard analysis.[18] At first, we investigated the binding performance of P_{RBZH}, the experiments were carried out by varying the concentration of *R*-BZH from 0.20 mM to 3.0 mM in acetonitrile in the presence of fixed amount of P_{RBZH}. The obtained binding data were plotted according to the Scatchard equation: $Q/[RBZH] = (Q_{max} - Q)/K_d$, where K_d is an equilibrium dissociation constant and Q_{max} an apparent maximum number of binding sites. As shown in Fig. 3, the Scatchard plot was not linear, indicating that the binding sites in P_{RBZH} were heterogeneous in respect to the affinity for *R*-BZH.

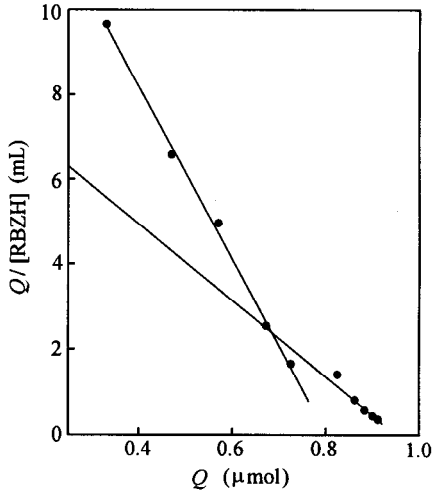


Fig. 3 Scatchard plots to estimate the binding characteristics of P_{RBZH}. Q: Amount of *R*-BZH bound to 20.0 mg of P_{RBZH}; $V = 2.0$ mL; $T = 25$℃; adsorption time: 12 h.

Because there were two distinct sections within the plot which can be regarded as straight lines, it revealed that two classes of binding sites were mainly produced in the P_{RBZH} in the studied concentration range of *R*-BZH. The $K_{d,1}$ and $Q_{max,1}$ of the higher affinity binding sites could be calculated to be 4.8×10^{-5} mol/L and 40.0 (mol/g of dry polymer from the slope and interception of its Scatchard plot. By the same treatment, $K_{d,2}$ and $Q_{max,2}$ of the lower affinity binding sites were 1.1×10^{-4} mol/L and 47.5 μmol/g of dry polymer. However, for the molecularly imprinted polymers containing different binding sites, there seem to be some approximate treatments in Scatchard analysis. The method does not consider the contribution of the lower affinity binding sites to the binding capacity of MIPs at low concentrations of substrates in the determination of higher affinity binding parameters. Similarly, at a high concentration of substrates, binding capacity of higher affinity binding sites is ignored in the determination of lower affinity binding parameters. So the binding parameter values obtained by Scatchard analysis are less accurate in these systems. In order to overcome the insufficiency of Scatchard analysis, the mathematical model of many independent classes of binding sites was introduced into molecularly imprinted polymers.[19] Suppose m classes of different binding sites exist in a molecularly imprinted polymer, which will be denoted P1, P2, $\cdots\cdots P_m$. Then the reaction between P_i and substrate S may be written as

$$S + P_i = SP_i \quad (1)$$

If each reaction proceeds independently to equilibrium, the following equation can be obtained

$$K_{d,i} = C(Q_{max,i} - Q_i)/Q_i \quad (2)$$

In Eq. (2), $K_{d,i}$ is equilibrium dissociation constant of i class of binding reaction and $Q_{max,i}$ an apparent maximum number of i class of binding sites, C the free concentration of substrate S and Q_i the equilibrium binding amount of i class of binding sites. From Eq. (2), Q_i can be expressed as

$$Q_i = Q_{max,i}C/(K_{d,i} + C) \quad (3)$$

If Q denotes the total binding amount of m classes of binding sites, then Q can be expressed as

$$Q = \sum Q_i = \sum Q_{max,i}C/(K_{d,i} + C) \quad (4)$$

For P_{RBZH}, Scatchard analysis showed that two classes of binding sites existed, that is, $m = 2$. Suppose $m = 2$, the obtained experimental points were fitted by Eq. (4). The resulting fitting curve was in good agreement with the experimental points as shown in Fig. 4.

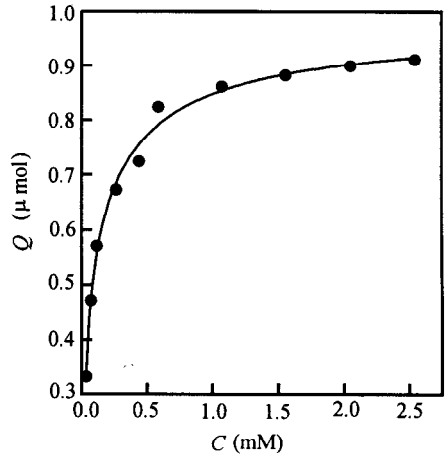


Fig. 4 Fitting curve obtained by Eq. (4). Q is the amount of *R*-BZH bound to 20.0 mg of P_{RBZH}.

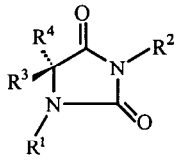
$K_{d,1}$, $K_{d,2}$, $Q_{max,1}$ and $Q_{max,2}$ obtained by the final parameter estimates were 3.5×10^{-5} mol/L, 4.3×10^{-4} mol/L, 31.5 μmol/g, and 17.5 μmol/g, respectively. Because the treatment is not limited by the substrate concentration used in the experiments, we think that the values obtained by the model are more reasonable. The result is in agreement with the above prediction of the binding characteristics of the polymer.

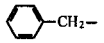
Selectivity of P_{RBZH}

Using the imprinted polymer P_{RBZH} as recognition matrix and the compounds of 5 closely related hydantoin-structures as tested substrates (Table 1), a substrate- and enantio-selectivity study was undertaken by batch methods.

The determined amounts bound to P_{RBZH} and P_0 were shown in Fig. 5.

Table 1 Molecular structures of some hydantoin compounds



Compounds	R^1	R^2	R^3	R^4
R-BZH	H	H		H
S-BZH	H	H	H	
DPH	H	H		
MDPH	H	CH_3		
DMDPH	CH_3	CH_3		

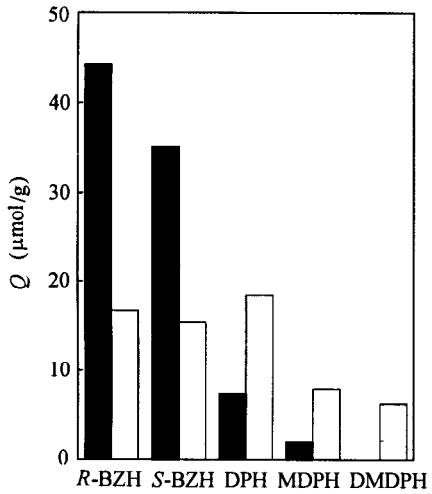


Fig. 5 Selectivity of the imprinted polymer P_{RBZH}. Concentration of substrates: 2.0 mmol/L; V = 2.0 mL; T = 25℃; Polymers: 20.0 mg; adsorption time: 12 h; black: P_{RBZH}; white: P_0; solvent: acetonitrile.

Fig. 5 showed that P_{RBZH} exhibited good selectivity for *R*-BZH as compared with all the other tested compounds. P_0 exhibited low binding amounts for any of the substrates. The evidences indicate that the imprinting method creates a micro-environment based on shape selection and position of functional groups that recognizes the best *R*-BZH template molecules. The obvious difference of binding selectivity between P_{RBZH} and P_0 mainly results from the amide groups within microcavities created in P_{RBZH} network by molecular imprinting. P_{RBZH} may interact with the *R*-BZH template molecule by two amide groups in a cavity. For its enantiomer *S*-BZH, P_{RBZH} could only interact with it by one amide group in a cavity due to the lack of complementarity to the functional groups between *S*-BZH and P_{RBZH}. So P_{RBZH} showed much higher binding capacity for *R*-BZH than for *S*-BZH and exhibited good enantiomeric recognition. For other tested substrates, owing to their greater spatial structures, it is difficult to accommodate these molecules in the imprinted cavities. Thus P_{RBZH} showed little or no binding capacity for them. Although P_0 has the same chemical composition as P_{RBZH}, the arrangement of amide groups in P_0 is random. So it would relatively poorly bind any of the tested compounds by weak non-specific adsorption and showed no selectivity for them. These results suggest that P_{RBZH} is capable of selectively recognizing *R*-BZH from the hydantoin derivatives. In comparison, the non-imprinted control polymer P_0 shows no selectivity. Considering the results, it may be possible to employ this polymer as recognition matrix in combinatorial screening protocols.

Effect of water on enantioselectivty of P_{RBZH}

The above studies suggested that P_{RBZH} exhibited substrate- and enantio-selectivity for *R*-BZH in acetonitrile. Thus we decided to examine the effect of water on the enantioselectivity of P_{RBZH} in the equilibrium binding system. Acetonitrile /water was used as the solvent. In 0-30% of the content of water, the binding amounts of P_{RBZH} for *R*- and *S*-BZH were determined and the average values of triplicate independent results were used in Fig. 6.

When the content of water in bound solutions increased from 0 to 15%, it was found that the binding amounts of P_{RBZH} for *R*- and *S*-BZH (Q) decreased dramatically and Q for *R*-BZH changed more rapidly than Q for *S*-BZH (Fig.6). Hence, enantiomeric separation factor α was also significantly reduced (Fig.6). At 15%(V/V) water in the solution, the difference between its binding capacity of the polymer and that of P_0 was very small, the polymer had lost its enantioselectivity for *R*-BZH and the value of α was 1.05. These results can be explained that the specific recognition is achieved by hydrogen bonding interactions between the sample molecules and the recognition sites of P_{RBZH} in the organic solvent; the addition of water to the bound solutions decreases this recognition because of the water

molecule acting as competing ligands for the hydrogen bonding sites. When the content of water increases further, the binding amounts of P_{RBZH} for *R*- and *S*-BZH also begin to increase due to the increase of hydrophobic interactions, however its separation factor α has little or no enhancement. These results further prove that hydrogen bonds are the dominant interactions for the recognition of P_{RBZH} for *R*-BZH.

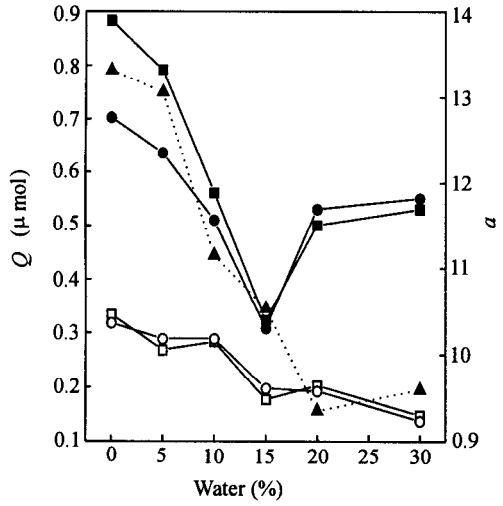


Fig. 6 Effect of water on the binding amount and the enantiomeric separation factor of P_{RBZH}. Concentration of *R*- and *S*-BZH: 2.0 mmol/L; $V = 2.0$ mL; $T = 25℃$; adsorption time: 12 h; polymers 20.0 mg; dot line: α vs. water(%), $\alpha = Q_R C_S / Q_S C_R$. Q_R and Q_S: amounts of *R*- and *S*-BZH bound to P_{RBZH}, C_S and C_R: free concentrations of *R*- and *S*-BZH in bound solutions. Solid square: P_{RBZH}. + *R*-BZH; open square: P_0 + *R*BZH; solid dot: P_{RBZH} + *S*-BZH; open dot: P_0 + *S*-BZH. Triangle: α.

Conclusions

We have synthesized and characterized an imprinted polymer selective for *R*-BZH insoluble in non-polar solvents by using acrylamide as hydrogen bonding functional monomer in a polar solvent. The preliminary results obtained indicated that highly specific recognition sites could be created within the synthetic polymer . Good substrate and enantiomeric recognition were obtained using hydrogen bonding interactions. The polymer can make up for the inadequacy of MAA-based imprinting polymers. The results may be applied for the development of selective separation methodology in the molecular imprinting field.

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