THE ANALYST
FULL PAPER
www.rsc.org/analyst

Systematic study of steric and spatial contributions to molecular recognition by non-covalent imprinted polymers

David A. Spivak* and Jason Campbell

Louisiana State University, Department of Chemistry, Baton Rouge, LA 70803, USA

Received 21st December 2000, Accepted 5th March 2001
First published as an Advance Article on the web 10th April 2001

Although molecular imprinting is a widely accepted method for producing template specific polymers, the general rules for prediction and control of the binding and catalytic properties of these materials are still not fully understood. One reason for this is the problematic structural analysis of the active sites in the polymers, which are not amenable to X-ray crystallography or microscopic techniques due to their amorphous and heterogeneous nature. Therefore, molecular probes have been the most informative agents for the analysis of the structure of active sites. This paper focuses on the steric and geometrical aspects of shape recognition in non-covalent imprinted polymers, with particular effort to minimize other factors contributing to molecular recognition by the polymers. Chiral amine compounds with systematic changes in spatial, distal and conformational components of sterically controlled molecular recognition were investigated for use as non-covalent imprinted polymers. Chromatographic studies revealed that steric and spatial interactions influence the selectivity properties of imprinted polymers in a predictable fashion.

Introduction

Molecular imprinting is an established method for the production of polymeric artificial receptors for specific molecular recognition. The ease of this method makes it competitive with other synthetic organic methods of molecular recognition. Although biomolecules often provide superior molecular recognition properties, imprinted polymers provide easier methodology, greater stability and compatibility with organic solvents, high temperatures and high pressures. Furthermore, the polymer network itself provides immobilization of binding sites on a solid support that can be utilized in many applications.

Molecular imprinting can be performed using two approaches: one employing functional monomers covalently attached to the template, and the other utilizing non-covalent interactions between functional monomers and template. Non-covalent imprinting has become the method of choice due to its facile preparation and the tendency for non-covalent imprinted polymers to outperform covalent imprinted polymers. Schemes such as that shown in Fig. 1 are helpful to convey the concept of molecular imprinting; however, some of the figures may not necessarily reflect the true nature of the process. There are essentially three steps to this method: (i) formation of the pre-polymer complex (PPC); (ii) copolymerization of PPC with cross-linking monomers to form a network polymer incorporating the functional monomers; (iii) removal of the template. The resulting polymer is postulated to contain binding cavities that are complementary in shape and functionality to the template molecule. Validation of the formation of specific binding sites in polymers made using this method is performed primarily by binding studies that demonstrate a preference for binding of the template molecule over other related molecules.

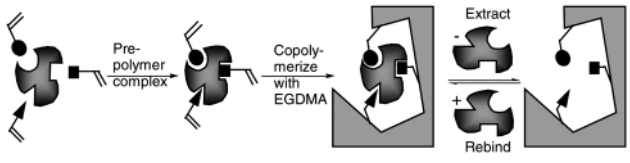


Fig. 1 Outline of the molecular imprinting strategy.

The literature has experienced a tremendous increase in the number of examples in which the method of molecular imprinting has been successful for the specific binding of targeted molecules.[1] The motivation behind a large number of these reports has been applications oriented towards the development of chromatographic supports,[2] chiral separation media,[3] solid phase extraction materials[4] and sensors.[5] The effectiveness of the imprinted polymer materials is often demonstrated by comparison of binding of the template *vs.* molecules with similar features, affording information on the extent of 'cross-reactivity' by the polymers.[6–9] These reports have also garnered important structure-binding relationships between specificity and atomic composition, topography and shape of the template molecule. The following list provides a number of architectural elements that have been shown to contribute to the substrate binding strength and specificity of imprinted polymers: (i) the number of interactions between the functional monomer(s) and the template; (ii) the innate binding strength of the functional monomer(s) for the template; (iii) the nature of the non-covalent interactions employed [hydrophobic, solvatophobic; electrostatic (ion–ion, ion–dipole, dipole–dipole); hydrogen bonding; dispersion and induction forces; π–π stacking (face–face interaction); charge transfer interactions]; (iv) the cooperativity of template substructures towards binding; (v) the size of the template (sterics); (vi) the shape of the template; (vii) the spatial relationships between template substructures; (viii) the distal relationships between template substructures; (ix) the conformational flexibility of the template.

The size of the list gives some indication of the difficulty of forming a general set of rules for predicting binding behavior in molecularly imprinted polymers (MIPs). Although a number of caveats have been put forward for the design of MIPs, the extent to which these individual factors contribute to MIP selectivity has not been fully established. In an effort towards the development of a deeper understanding of template effects on the specificity of MIPs, we present a systematic study of enantioselectivity in MIPs using chiral substituted amines. The focus of this study is on the effect of steric, distal and conformational factors on chiral selectivity by non-covalent MIPs.

DOI: 10.1039/b010233o

The chiral resolution of small molecules is one of the most important challenges in separation science, and of immense importance to pharmaceuticals and many other fields of chemistry.[10] Moreover, one of the best molecular probes for cavity shape in MIPs is the enantiomer. The reason for this is that all the physical properties of enantiomers are the same except for the three-dimensional positioning of atoms in space; consequently, discrimination of enantiomers can be viewed as a geometrical phenomenon. Therefore, it is of interest to employ enantiomers to investigate the effect of geometric variables such as sterics and their distal relationship to the formation of cavity shape and substrate recognition in imprinted polymers. Steric interactions are often considered to be the most definitive in determining the selectivity of a receptor for a substrate, since two molecular moieties cannot occupy the same space. For MIPs, this is a good place to start evaluating the selective nature of the binding cavity. The extent to which the selectivity has been governed by cavity shape has been reported in a few cases for covalently bound templates.[11–13] This work is particularly informative with regard to selectivity due to template shape, since contributions of binding energy to selectivity were not a factor in these examples in view of the fact that covalent rebinding was used. Several non-covalent systems have been reported that have an element of shape selectivity to them; however, the extent of geometric and steric interactions is not well known, since they are often accompanied by other factors contributing to molecular recognition.[8,9,14]

There is an intimate relationship between binding affinity and geometrical considerations in determining substrate specificity. This includes shape selectivity and pre-organization of binding groups. If we limit a molecular probe to essentially one type of binding group, we can then neglect contributions of binding energy to selectivity and focus on geometrical considerations of shape selectivity for specific binding. For example, the steric influence of differently sized groups surrounding a chiral carbon center on molecular recognition by MIPs can be tested. It has been pointed out, however, that one difficulty in creating complementary shaped cavities to small molecules may be that the monomers used are about the same size as the templates.[15] Thus a high resolution complementary surface might not be expected. Large differences in size between small, medium and large groups may lead to improved resolution. Furthermore, if sterics could be used to adjust the stereochemical recognition of a compound with one stereocenter, it would be of interest to explore the steric effects of two chiral centers on molecular recognition by imprinted polymers.

In addition to steric considerations, another important factor is the distance of a molecule's discriminating features from the binding group interaction with the polymer. The use of enantiomers with one chiral group as the discriminating feature provides a good molecular probe of this effect. Investigation into the resolving power of imprinted polymers with different distances between the primary binding event and the chiral center could reveal a distance–geometry algorithm for molecular recognition. Last, an important consideration is the conformational entropy of both the substrate and the polymeric receptor. For MIPs, there are three sources of conformational entropy to be considered. First, conformational flexibility arises from the cross-linked matrix, which is primarily regulated by the amount of cross-linking.[15–17] A second source of conformational entropy is from the functional monomer. This has been reported as a controlling factor for imprinted polymers, where an inverse relationship between the flexibility of the binding site functional group and chiral resolution was observed.[18] In this report, we focus on a third important area of conformational entropy, the conformation of the substrate molecule on rebinding to the polymer. NMR and modeling studies on molecular imprinting targets have qualitatively ascribed some recognition effect to differences in conformation.[17,19] Another study comparing imprinted molecules with varying rigidity found that binding interactions dominated any contribution by conformational rigidity.[20]

Experimental

General

Ethylene glycol dimethacrylate (EGDMA, Polysciences), as received, was distilled *in vacuo* (94 °C) over boiling chips prior to polymerization. Methacrylic acid (MAA, Aldrich) was distilled over CaH_2 (80 °C). *R*- and *S*-enantiomers of α-methylbenzylamine, β-methylphenethylamine, 1-(1-naphthyl)ethylamine, bis(α-methylbenzyl)amine, bis[(1-naphthyl)ethyl]amine, 1-aminoindane and 2,2'-azobisisobutyronitrile (AIBN) were all purchased from Aldrich Chemicals and were used without further purification. All solvents were obtained from commercial suppliers, and were purified prior to use.

Polymer preparation

The following procedure was used for all imprinted polymers. In a borosilicate scintillation vial, 1.28 mmol of the *S*-enantiomer of the chiral amine was dissolved in 8.0 mL methylene chloride. To this solution was added 5.0 g EGDMA (25.2 mmol), 0.53 g MAA (6.3 mmol) and 0.11 g AIBN (0.64 mmol). The control polymer was formulated in a similar fashion, without introduction of a template molecule. The solution was purged by bubbling nitrogen gas into the mixture for 5 min, and then capped and sealed with Teflon tape and parafilm. The samples were inserted into a photochemical turntable reactor (ACE Glass Inc.) which was immersed in a constant temperature bath. A standard laboratory UV light source (a Canrad-Hanovia medium pressure 450 W mercury arc lamp), jacketed in a borosilicate double-walled immersion well, was placed at the center of the turntable. The polymerization was initiated photochemically at 20 °C and the temperature was maintained by both the cooling jacket surrounding the lamp and the constant temperature bath holding the entire apparatus. The polymerization was allowed to proceed for 10 h, and was then used for the chromatographic experiments. It should be noted here that the ratio of MAA to print molecule was 4 : 1 which has been found to be the optimum in other investigations of imprinted polymers.[9] We also investigated polymers incorporating an MAA : α-methylbenzylamine ratio of 1 : 20 and found absolutely no enantioselectivity in these polymers.

Chromatographic experiments

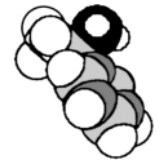
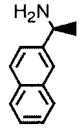
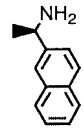
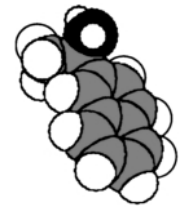
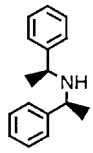
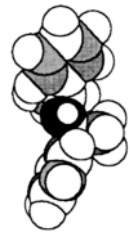
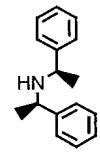
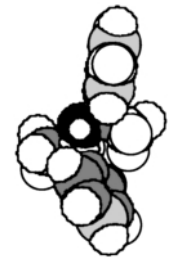
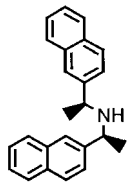
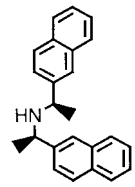
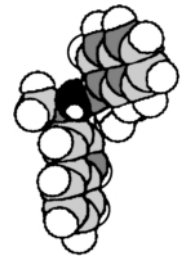
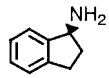
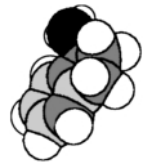
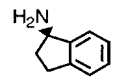
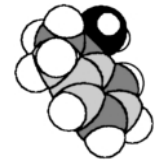
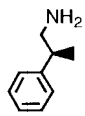
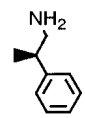
The polymers were ground using a mortar and pestle; the particles were sized using USA Standard Testing Sieves (VWR) and the fraction between 20 and 25 μm was collected. The particles were slurry packed, using a Beckman 1108 Solvent Delivery Module, into stainless steel columns (length, 10.0 cm; id, 4.6 mm) to full volume (approximately 0.6 g of polymer) for chromatographic experiments. The polymers were then washed online for 12 h using acetonitrile–acetic acid (90 : 10) at a flow rate of 0.2 mL min^{-1} to remove the template. HPLC analyses were performed isocratically at room temperature (21 °C) using a Hitachi L-7100 pump with a Hitachi L-7400 detector. The flow rate in all cases was set at 1.0 mL min^{-1} using a mobile phase consisting of acetonitrile–acetic acid (90 : 10). Sample injections were 1.5–10 μL of a 10 mM solution of amine in acetonitrile. The void volume was determined using acetone as an inert substrate. The separation factors (α) were measured as the ratio of capacity factors (k'_S/k'_R). The capacity factors were determined by the relation $k' = (R_v - D_v)/D_v$, where R_v is the retention volume of the substrate and D_v is the void volume.

Results and discussion

A previous study had established that α-methylbenzylamine could be chromatographically resolved on an imprinted polymer using only acetonitrile as the elution solvent,[21] whereas we found it necessary to use a mobile phase of higher eluent strength. The difference in the binding affinity may be related to the fact that the previously made polymer was prepared under thermal conditions, whereas we have utilized photochemical polymerization at ambient conditions. A number of other aryl amine compounds, in addition to α-methylbenzylamine, were imprinted in this study to further examine the molecular recognition properties in imprinted polymers. The molecular probes chosen for this study are shown in Table 1, which also provides energy minimized space filling models for both enantiomers in order to obtain a clearer view of the steric and distal contributions of the different chiral amines used.

The choice of amine functionality was natural for reliable interactions with MAA for the formation of the PPC and rebinding studies.[22] An important common feature of all the chiral amine compounds used in this study is that there is only one electrostatic binding interaction afforded by all substrates. This is important since the generally accepted Ogsten model for enantioselectivity requires three points of contact between substrate and receptor.[23] Therefore, enantioselectivity exhibited by the polymers will be due to at least two other interactions, most likely steric in origin. However, at least one attractive force, such as the single electrostatic interaction in this case, is necessary, otherwise there will be no binding and, consequently, no enantioselectivity obtained. The initial attraction of

Table 1 Structure of chiral amines used for molecular imprinting, and space filling models illustrating the minimized structures for each enantiomer using MOPAC on CHEM 3D [MOPAC is a semi-empirical calculation supplied with the CambridgeSoft CS ChemOffice Pro software package (CambridgeSoft Corporation, 100 Cambridge Park Drive, Cambridge, MA 02140, USA). Energy minimization employed AM1 theory with minimum RMS gradient set to 0.100]

Entry	*S*-Enantiomer	*S*-Enantiomer	*R*-Enantiomer	*R*-Enantiomer
1 α-Methylbenzyl amine				
2 1–(1–Naphthyl) ethylamine				
3 bis(α-Methyl benzyl)amine				
4 bis[(1–Naphthyl) ethyl]amine				
5 1–Aminoindane				
6 β-methylphenethyl amine				

the amine group to the carboxylate groups on the polymer can then be considered the primary binding event. The presence of the PPCs was verified by ^{1}H NMR titrations of the solution phase complexes in chloroform for the protons located α to the amine group. Fig. 2 shows the change in the ^{1}H NMR shifts, $\Delta\delta$, as the ratio of MAA : template is increased from 0 to 7. For all amine templates, the figure shows shift changes leveling off when up to four or seven equivalents of MAA are added. Since the ratio of MAA : template used in the polymer mixtures was 4 : 1, the NMR shifts would indicate that all the amines were complexed in the pre-polymer solution.

Imprinted polymers were synthesized using the *S*-enantiomer of each chiral amine. The specificity of the polymers was then investigated using HPLC, by obtaining capacity factors (k') for both the *R*- and *S*-enantiomers of each amine, on each of the imprinted polymers. The enantioselectivity by the polymers was evaluated by comparison of the separation factors, α ($\alpha = k'_S/k'_R$). A complete listing of all α values is shown in Table 2. In all cases, the best separation was achieved when the enantiomers of the print molecule were applied to the column. The fact that the memory effect of the imprinting method is responsible for the chiral recognition is indicated by the lack of specificity seen for any of the enantiomers on the blank polymer. Furthermore, the significant differences in the chiral selectivities of the polymers cannot be accounted for by differences in ionic interactions, since the pK_a values for the different amines are all within the same range of 7.1–9.2 as shown in Table 3.

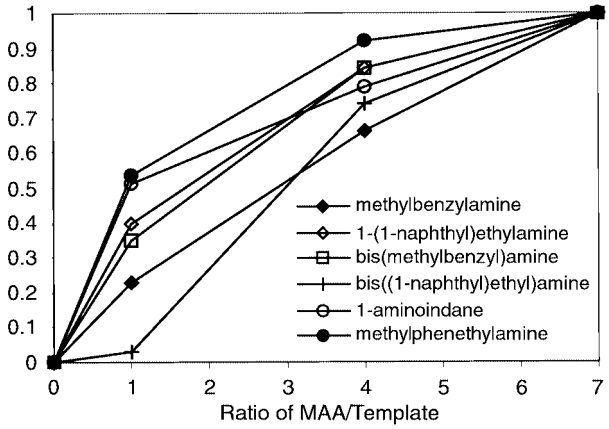


Fig. 2 ^{1}H NMR titration curves for various MAA : template ratios in chloroform.

Focusing first on the steric contributions of groups bound to the chiral center of the imprinted molecule, the polymer imprinted with α-methylbenzylamine was compared with the polymer imprinted with 1-(1-naphthyl)ethylamine. The separation factors of the polymers for their own substrates were 1.33 and 1.58, respectively, showing an improvement of chiral resolution upon increasing the aryl group size by approximately two-fold. However, a significant level of cross-reactivity was found, indicated by a separation factor of approximately 1.2 for both polymers with the opposite substrate, showing a relatively high tolerance between these two structures. This was the only cross-reactivity seen for polymers imprinted with α-methylbenzylamine. This is in contrast to the polymer imprinted with 1-(1-naphthyl) ethylamine, which showed cross-reactivity in all cases except for the polymer imprinted with 1-aminoindane and the control. This trend may imply that the naphthalene moiety may serve as a 'privileged' structure for imprinted polymers, *i.e.* one that is especially good as a generic

Table 2 Separation factors (α) for the different chiral amines on each imprinted polymer

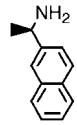
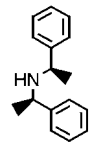
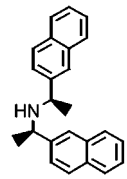
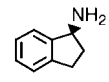
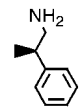
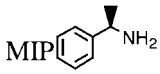
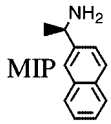
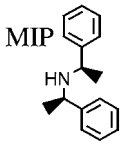
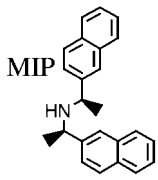
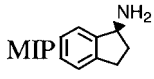
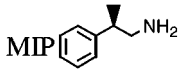
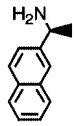
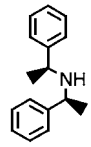
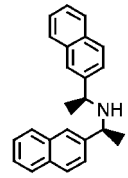
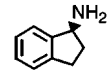
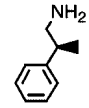
MIP's \ Substrates						
	**1.33**	1.20	1.00	1.00	1.00	1.00
	1.23	**1.58**	1.14	1.16	1.00	1.07
	1.00	1.00	**2.26**	1.78	1.00	1.00
	1.00	1.00	1.65	**3.25**	1.00	1.00
	1.05	1.03	1.00	1.05	**1.44**	1.01
	1.00	1.01	1.00	1.00	1.00	1.00
Blank	1.00	1.00	1.00	1.00	1.00	1.00

Table 3 Amine group pK_a's for templates used in this study[a]

Template						
pK_a	8.5	7.6	7.1	N/A	8.0	9.2

[a] p*K*a data for bis[(1-naphthyl)ethyl]amine was not available due to lack of solubility in water or reliable combinations of acetonitrile or acetone and water.

steric building block for chiral selectivity of a number of different molecular structures by imprinted polymers.

The polymer imprinted with bis(α-methylbenzyl)amine has two identical chiral centers, essentially doubling the recognition interactions possible in the case of α-methylbenzylamine. However, there is no cross-reactivity seen between these two polymers and substrates, indicating that there is little relationship in the recognition of these substrates by MIPs. The bis-substituted amines are most likely sterically excluded from the binding sites of the mono-substituted amines; however, the lack of recognition of the mono-substituted amines by the bis-substituted amine imprinted polymers is a little more complicated. The reason for this may be due in part to the different conformers of the print molecules, resulting in recognition sites for different shapes in combination with different spatial organization.[19] This can be seen from a visual comparison of the structures in Table 1. Taking a one-point binding interaction as a model, one can postulate that a difference in the equilibrium constant of the two enantiomers takes place in the chiral binding site, arising from the site forcing one of the enantiomers to take an unfavorable conformation. Furthermore, the three-dimensional steric interactions of the polymer matrix surround the amine primary binding event in a much more complex fashion than with the single stereocenter analogs. The complexity should increase the selectivity for the bis-substituted amines *vs.* mono-substituted amines, since there are fewer degenerate states that would accommodate the steric groups in alternative geometries. Last, since the monomers are of approximately the same size as the imprint molecules, the greater steric bulk of the bis-substituted species may afford a more optimal spatial fit.[15] This would explain why a greater separation factor was found for the bis[(1-naphthyl)ethyl]amine enantiomers on their imprinted polymer *vs.* the separation found for the bis(α-methylbenzyl)amine enantiomers on the polymer imprinted with (*S*,*S*)-bis(α-methylbenzyl)amine.

In an effort to assess the effect of the conformational entropy of the imprinted molecule on molecular recognition, we imprinted 1-aminoindane as a conformationally 'locked' model of α-methylbenzylamine. Table 2 shows that there is relatively little chiral recognition found for α-methylbenzylamine on the 1-aminoindane imprinted polymer, and no stereoselectivity seen for 1-aminoindane on the α-methylbenzylamine imprinted polymer. These two molecular compounds may not share the same optimum conformation which precludes cross-reactivity. However, the separation factor for 1-aminoindane on its imprinted polymer was higher than that found for α-methylbenzylamine on its imprinted polymer, possibly related to increased specificity from a decrease in conformational entropy that would increase the energy of binding. Finally, the effect of distance between the primary binding event and the chiral center can be seen from a comparison of the separation factors found for polymers imprinted with α-methylbenzylamine and β-methylphenethylamine. Moving the primary binding event further away from the chiral center was anticipated to lower the stereoselectivity by the MIP. Table 2 shows that an increase in distance of the amine from the chiral center does in fact decrease the extent of enantiomer recognition. The polymer imprinted with β-methylphenethylamine gave a separation factor of only 1.13 compared to a separation factor of 1.33 for the α-methylbenzylamine imprinted polymer. Conformational flexibility may also play a role in the loss of recognition in that the point of binding to the amine may afford the closest contact for the longest period of time. In this case, all substituents held close to the point of contact would be most susceptible to steric interaction with the polymer binding site. Thus, positive interactions between the correct substrate and polymer would be maximized leading to optimal recognition, and repulsion interactions of the wrong substrate and polymer would be maximized, limiting the binding interaction. This effect would be lessened in both cases the further the initial point of contact is from the spatial differentiation of the molecules.

References

1 For a recent review, see G. Wulff, *Angew. Chem., Int. Ed.*, 1995, **34**, 1812.
2 V. T. Remcho and Z. J. Tan, *Anal. Chem.*, 1999, **71**, 248A.
3 B. Sellergren, *Pract. Approach Chiral Sep. Liq. Chromatogr.*, ed. G. Subramanian, VCH, Weinheim, 1994, pp. 69–93.
4 M. C. Hennion, *J. Chromatogr., A*, 1999, **856**, 3.
5 K. Haupt and K. Mosbach, *Chem. Rev.*, 2000, **100**, 2495.
6 V. P. Joshi, M. G. Kulkarni and R. A. Mashelkar, *J. Chromatogr., A*, 1999, **849**, 319.
7 E. O. Dela Cruz, H. Muguruma, W. I. Jose and H. Pederson, *Anal. Lett.*, 1999, **32**, 841.
8 T. P. O'Brien, N. H. Snow, N. Grinberg and L. Crocker, *J. Liq. Chromatogr. Relat. Technol.*, 1999, **22**, 183.
9 L. I. Andersson, D. J. O'Shannessy and K. Mosbach, *J. Chromatogr.*, 1990, **513**, 167.
10 S. G. Allenmark, *Chromatographic Enantioseparation Methods and Applications*, Ellis Horwood Ltd., Chichester, 1988.
11 K. J. Shea and D. Y. Sasaki, *J. Am. Chem. Soc.*, 1989, **111**, 3442.
12 G. Wulff, W. Best and A. Akelah, *React. Polym.*, 1984, **2**, 167.
13 G. Wulff and E. Lohmar, *Isr. J. Chem.*, 1979, **18**, 279.
14 B. Sellergren, *ACS Symp. Ser.*, 1998, **703**, 49.
15 G. Wulff, *ACS Symp. Ser.*, 1986, **308**, 186.
16 D. A. Spivak and K. J. Shea, *J. Am. Chem. Soc.*, 1997, **119**, 4388.
17 B. Sellergren, M. Lepisto and K. Mosbach, *J. Am. Chem. Soc.*, 1988, **110**, 5853.
18 G. Wulff and H.-G. Poll, *Makromol. Chem.*, 1987, **188**, 741.
19 M. Lepisto and B. Sellergren, *J. Org. Chem.*, 1989, **54**, 6010.
20 W. Jinfang, Z. Liangmo, M. Zihui, L. Xueliang, W. Qinghai and Z. Daoqian, *Chin. J. Anal. Chem.*, 1999, **27**, 1420.
21 Z. H. Meng, L. M. Zhou, Q. H. Wang and D. Q. Zhu, *Chin. Chem. Lett.*, 1997, **8**, 345.
22 D. A. Spivak and K. J. Shea, *J. Org. Chem.*, 1999, **64**, 4627.
23 A. G. Ogsten, *Nature*, 1948, **162**, 963.