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Review

Highly selective separations by capillary electrochromatography: molecular imprint polymer sorbents

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Abstract

Molecular imprint polymers (MIPs) are synthesized in the presence of a template, or 'imprint' molecule which results in the formation of specific recognition cavities complementary to the template in shape and chemical functionality. The resultant MIP then acts as a selective binding medium for the template molecule. The utility of MIPs lies in the selectivity of the rebinding process, which is based on molecular recognition. In many cases, the selectivity achieved with MIPs toward a particular molecule is comparable to that observed with antibodies. This has led to the application of MIPs to several areas of analytical chemistry including immunoassays, sensors and separations media. One of the most successful application areas of MIPs has been as chromatographic sorbents, where they have been utilized predominately in chiral separations. The use of MIP sorbents in CEC is attractive in that it combines the selectivity of a molecular recognition process with the enhanced flow dynamics of CEC, which can result in higher efficiency and shorter analysis times. This paper will review the use of molecular imprinted stationary phases in CEC. Following a brief introduction to molecular imprinting, various methodologies for preparation of MIP-CEC capillaries in addition to applications of the technique will be discussed.

Keywords: Reviews; Molecular imprint polymers; Stationary phases, CEC

Contents

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1. Introduction

Molecular imprinting is a technology in which specific recognition sites are formed in a polymer matrix by synthesis in the presence of a template molecule. The imprinted polymer is capable of selectively rebinding the template molecule through a host–guest type interaction. Although the foundations of the molecular imprinting technique were established decades ago [1,2], the technique is enjoying a resurgence of research interest. The highly selective recognition properties of molecular imprint polymers (MIPs) rival those of natural biological species, such as receptors and antibodies. However, MIPs possess several advantages over their biological counterparts including low cost, ease of preparation, and good physical and chemical stability. In addition to their widespread use as selective stationary phases in separations, MIPs have found use as synthetic mimics of natural biological selectors in a number of other applications in including sensors [3], immunoassays [4] and catalysis [5]. Here a cursory overview of molecular imprinting will be given. For a more detailed treatment, the reader is referred to a number of thorough literature reviews on the subject [6–9].

1.1. Principles of molecular imprinting

In molecular imprinting, a highly cross-linked organic polymer is synthesized in the presence of a template, or imprint, molecule. Prior to polymerization, functional groups on the template interact with those on the monomer(s). Depending on the method employed, these interactions are reversible covalent bonds or various non-covalent intermolecular forces. Polymerization begins usually by activation of a free radical initiator with UV radiation or thermally, at which point the polymer begins to form around the template molecule. The associative interactions between the template molecule and the monomers fix the three-dimensional orientation of functional groups on the MIP about the template. After polymerization is complete, the template is removed either by hydrolysis (covalent MIPs) or by mild extraction with an appropriate solvent (non-covalent MIPs), leaving cavities, or 'imprints' in the polymer that are complementary in shape and functionality to the template species.

MIPs are typically prepared with a molar excess of cross-linking reagent in order to impart structural rigidity, which enables the imprinted sites to retain their shapes and affords some resistance to swelling. The cavities formed through the imprinting process serve as recognition sites in which specific rebinding of the template molecule can occur. It is this recognition process that is the hallmark of MIPs.

The non-covalent approach to MIP synthesis is more widely used for preparing MIPs as chromatographic stationary phases than is covalent imprinting. Reasons for this include the greater versatility of non-covalent imprinting with respect to the available modes of interaction and the generally more favorable kinetic properties of the recognition process.

In non-covalent imprinting, methacrylic acid (MAA) is commonly used as a functional monomer. This reagent can act as a hydrogen bond donor or acceptor and has an acidic proton, enabling ionic interactions to be utilized in the imprinting process. Commonly used cross-linkers include ethylene glycol dimethacrylate (EDMA) and trimethylolpropane trimethacrylate (TRIM). In the pre-polymerization mixture, the template molecule can interact with the monomers via hydrogen bonding, ionic, $\pi-\pi$ and hydrophobic interactions. Hydrogen bonding and ionic forces are typically dominant. The effectiveness of these interactions is highly dependent upon the polarity of the medium, thus organic solvents of low polarity are used to obtain optimal imprints. In particular if hydrogen bonding is critical in the imprinting process, aprotic solvents are preferred to minimize competition by the solvent. The non-covalent imprinting process is depicted schematically in Fig. 1.

The advantages of MIPs, in particular high selectivity and physicochemical stability, have propelled the development of MIPs as chromatographic stationary phases, particularly in HPLC where they have been utilized mainly as chiral stationary phases (CSPs). In such applications, one enantiomer of the compound to be analyzed is employed as the template molecule. In the chromatographic separation, the template enantiomer is selectively retained on the MIP relative to its antipode.

Imprinted polymers as chiral stationary phases in

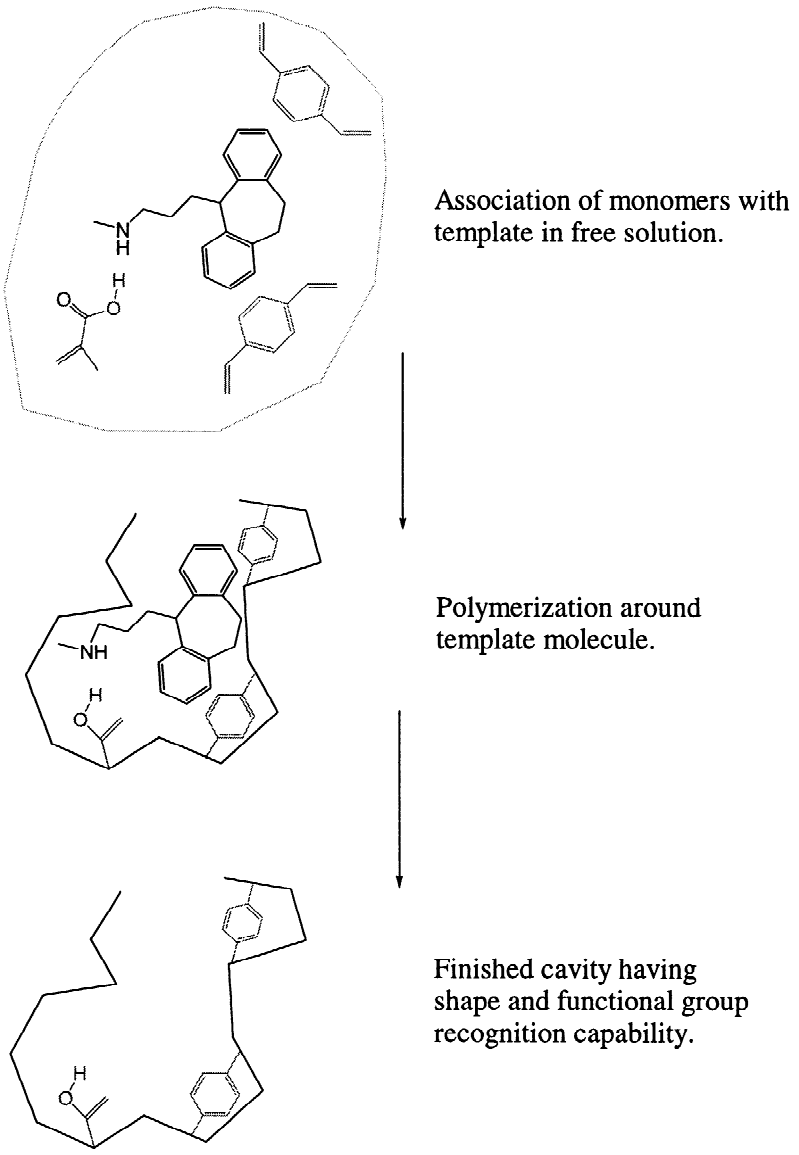

Fig. 1. A simplified representation of the synthesis of a noncovalent molecular imprint polymer.

HPLC generally yield higher enantioselectivities relative to conventional CSPs; values as high as 17.8 have been reported [10], although in certain instances MIP column provide less theoretical plates. Driven by this high selectivity, a variety of chiral compounds have been separated by MIP-HPLC including amino acid derivatives [11], β-blockers [12], non-steroidal anti-inflammatory drugs (NSAIDS) [13], peptides [14] and sugars [15]. An important advantage of MIPs relative to conventional chiral stationary phases is the ability to predetermine selectivity.

There are, however, limitations of MIPs as chromatographic stationary phases. The chromatographic behavior of the template molecule is usually characterized by severe tailing, which is believed to arise from a combination of factors: slow interaction kinetics between the template and the active site [16]

and the heterogeneous nature of the binding sites with respect to geometry and accessibility. Thus a nonlinear adsorption isotherm is typically observed for the template molecule on MIPs [17]. In practice, tailing can be ameliorated to some extent by conducting separations at elevated temperature and by gradient elution. Exacerbating this problem is the fact that much of the early work in developing MIPs for HPLC relied upon the bulk polymerization approach for MIP preparation (cf. Section 2), which yields irregularly shaped particles having a broad size distribution. These are unfavorable qualities from the standpoint of chromatographic efficiency. As a result, low plate numbers are obtained in most MIP-HPLC separations even for non-imprinted species. For the imprint molecule, the combination of these factors can lead to very poor efficiency. Although many successful separations of chiral compounds have been obtained owing to the tremendous selectivities of MIPs, in general poor efficiency and peak shape have limited achievable resolution.

In order to improve efficiency and increase resolving power of MIP columns, alternative methods have been investigated for developing uniformly sized MIP particles for HPLC. Some of these methods include dispersion polymerization [18], surface imprinting [19] and two step swelling or 'seed' polymerization [20]. Alternatively, other methods have been employed in which MIPs are prepared in situ as continuous rods, or monoliths [21]. The in situ methods are advantageous in that the need for column packing, often a time consuming and difficult process, is obviated. In CEC, the use of MIP based stationary phases is especially attractive owing to the technique's improved efficiency with respect to HPLC.

2. Methods of preparing MIP columns for CEC

2.1. Open tubular capillaries

In this approach, the MIP is prepared as a thin film, which is synthesized in situ and usually attached to the capillary wall by covalent bonds. This format provides the advantage of relatively simple capillary preparation because the MIP is prepared in situ. In addition, higher efficiencies are obtained in the open tubular format because the various dispersive contributions of packed beds are absent. However, this advantage comes at the expense of reduced sample capacity, which complicates detection.

Polyacrylate-based coatings have been successfully developed for reverse phase HPLC and CEC in the open tubular format [22]. This work was later adapted to produce wall-coated MIP stationary phases in 25-μm I.D. capillaries [23]. The procedure, which is outlined in Fig. 2, involved first functionalizing the capillary wall with a silane reagent containing an active double bond, 3-(trimethoxysilyl) propyl methacrylate, to provide anchoring points at which the MIP could attach. Following this treatment, the capillary was filled with a solution containing monomers (MAA and 2-vinyl pyridine), cross linker (EDMA), initiator (AIBN), template molecule and solvent. Polymerization was initiated thermally, after which a vacuum was applied to the capillary to evacuate residual solvent and to shrink the polymer into a thin film against the capillary wall. Baseline resolution of the D- and L-isomers of phenylalanine was achieved on a 25-μm I.D. MIP capillary with (10:1) v/v acetonitrile: 10 m*M* phosphate buffer as the mobile phase.

Using a variant of the dispersion polymerization technique, Brüggeman, et al. prepared wall-coated MIP capillaries for CEC [24]. After an initial derivatization step in which 3-methacryloxypropyl trimethoxysilane was reacted with activated surface silanols on the capillary wall, appropriate amounts of functional monomer (trans-3- (3-pyridyl)-acrylic acid), cross-linker, EDMA or divinylbenzene, initiator (azobis-cyclohexanecarbonitrile) in porogenic solvent (dimethyl sulfoxide) was flushed through the capillary. The thickness of the MIP films could be controlled by careful adjustment of the polymerization conditions.

2.2. Particulate-based media

2.2.1. Packed capillaries

A widely used method for preparing MIP columns for conventional scale HPLC is to obtain MIP particles of suitable size, which can then be slurry packed using procedures similar to those for conventional spherical HPLC packings. This is typically

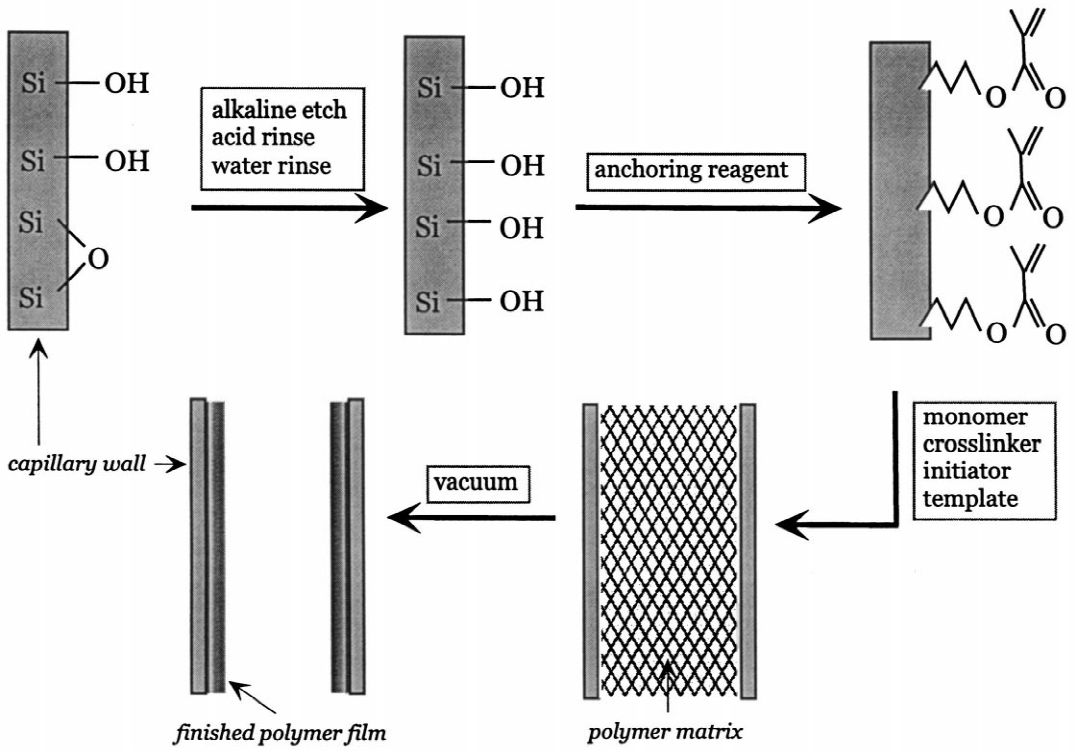

Fig. 2. Schematic diagram of a procedure used to make open tubular capillary columns for MIP-CEC.

accomplished by synthesizing the MIP in bulk, followed by grinding and sieving to yield particles with a nominal diameter of <30 μm. This approach has also been employed for preparing MIP capillaries for CEC.

Lin et al. [25] prepared MIPs using L-phenylalanine and its anilide derivative as templates. The bulk polymer was ground and sieved to a particle diameter of <10 μm and subsequently packed into capillaries for CEC using a slurry method. A short plug of polyacrylamide gel served as a retaining frit to hold the particles in the column.

Although relatively straightforward from the standpoint of the synthetic procedure, this approach has some rather serious limitations. One of these is a notable problem in packed column CEC in general: the need for retaining frits. These frits are difficult to fabricate and are often a major contributor to bubble formation within the capillary. The formation of gas bubbles in packed column CEC is a well-documented problem in CEC and leads to a disruption of current and EOF. Frits prepared from silicate materials, as is common practice in CEC, have been employed with MIP particles [26]. Although separations have been achieved, capillaries prepared in this manner are highly susceptible to bubble formation [26]. A possible source of this problem is large differences in zeta potential between the MIP particles and the frit material.

A further limitation of silicate-based retaining frits is the potential for adsorptive interactions between the analyte and frit material, which can result in tailing and loss of efficiency. For example, a protonated amine moiety on a molecule can undergo an ionic interaction with dissociated silanols on the frit surface.

The particles obtained by the bulk grinding and sieving method are highly irregular in shape with a relatively wide size distribution (Fig. 3). This, in conjunction with the intrinsically slow mass transfer

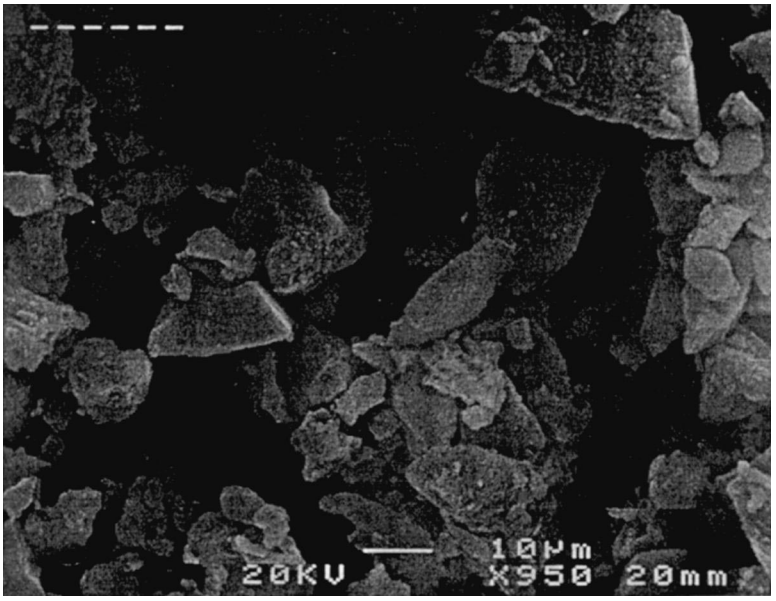

Fig. 3. SEM image of MIP particles obtained by grinding and sieving of bulk polymer.

and adsorption/desorption kinetics of MIPs serves to further limit efficiencies.

2.2.2. Entrapped MIP columns

In this approach, MIP particles are prepared (e.g. by grinding and sieving), packed into a capillary and immobilized by in an 'entrapment' medium, which is usually a network of polymer synthesized around the particles. The final structure comprises a packed bed supported by the network. One obvious advantage of this approach is that it obviates the need for retaining frits. In addition, the capillary can be cut to any desired length, providing an extra measure of versatility.

The first attempt at immobilizing MIP particles in a CEC capillary employed an acrylamide gel as the entrapment medium [27]. The MIP was prepared in bulk using L- phenylalanine as the template, ground and sieved to a particle size of ≤ 5 μm. The particles were subsequently combined with appropriate amounts of acrylamide and bisacrylamide in a mixture of acetonitrile and Tris buffer. A 75-μm I.D. capillary was filled with the mixture and placed in an oven at 40°C for 4 h to allow the gel to cure. Baseline separations of the phenylalanine isomers were achieved using 10–20% v/v acetonitrile in an aqueous Tris buffer as the eluent.

A more recent study employed a silicate-based entrapment network to immobilize the MIP [28]. This approach, which has also been used successfully to immobilize conventional silica ODS particles for CEC, involved first forming a temporary retaining frit at one end of the capillary. MIP particles, synthesized in bulk and sieved to <30 μm, were then slurry packed into the capillary. A mixture of potassium silicate and deionized water was flushed through the packed capillary, after which the capillary was placed in an oven for gradual heating from 40 to 160°C over a period of several days. The final steps were flushing with 0.1 *M* ammonium hydroxide, followed by drying at elevated temperature. CEC separations on these entrapped capillaries showed selective retention of the template species, dansyl-L-phenylalanine, relative to its D-isomer. The silicate entrapment medium did not appear to impede the molecular recognition process. An advantage of this approach is that it affords a mechanism to introduce surface charge density sufficient to sustain EOF independent of MIP synthesis. Unfortunately, the potential for adsorptive 'loss' of analytes, particu-

larly amines, increases substantially with the presence of the silicate network.

2.2.3. Dispersion polymerization

In this polymerization technique, the mixture of monomers, cross-linker, initiator and template molecule is prepared in a suitable porogenic solvent. The solution conditions are chosen such that the monomers are soluble, but the growing polymer chains are not. As the process continues, the polymer chains precipitate (a dispersion is formed) and form agglomerates several hundred nanometers to a few micrometers in diameter. Under the proper conditions, these agglomerates coalesce to form highly porous, spherical particles on the order of several μm diameter. This process can either be conducted in situ or externally. In the latter approach the particles are typically packed into columns using slurry techniques.

MIP particles synthesized by dispersion polymerization have been used successfully in HPLC [18]. The same general approach has been employed to prepare MIP capillaries for electrokinetic separations [29]. In this procedure pentamidine, a drug used in the treatment of AIDS-related pneumonia, was combined with MAA, EDMA and AIBN in 2-propanol. Addition of a small amount of water was necessary to optimize the mixture. A 100-μm I.D. capillary was then filled with the mixture and polymerization carried out thermally at 60°C for 24 h. Physical characterization of the polymer revealed particles approximately 10 μm in diameter, which comprised agglomerates of globular particles with diameters between 2 and 4 μm. The capillaries exhibited relatively low flow resistance; a pressure of 1300 p.s.i. was sufficient to flush mobile phase through 25-cm long capillaries.

2.3. Monoliths

As implied by the name, monolithic columns consist of one continuous porous cylinder or rod of stationary phase inside the column. There are several advantages to this approach. Unlike other methods, the polymer is synthesized in situ, avoiding the need for column packing and retaining frits and in addition, the amount of wasted material is reduced. As there are no discrete particles, efficiency in these media is dictated by pore size, which can be controlled by adjusting the composition of the porogenic solvent.

Svec and Fréchet presented an elegant method for preparing macroporous polymer columns for HPLC in 1992 [30]. The monolith was made sufficiently porous by means of a blended porogenic solvent consisting of (80:20) v/v cyclohexanol and decanol. This procedure was later adapted to prepare MIP columns for HPLC [21], which were used in separations of diaminonapthalene isomers and enantiomers of phenylalanine anilide. The porogen used in these procedures, a blend of cyclohexanol and decanol, was tailored to produce highly porous monoliths with sufficient permeability to flow. These protic solvents, however, are generally considered undesirable for non-covalent imprinting, in particular because they can disrupt hydrogen bonding interactions between the template and monomer(s).

This limitation was addressed by Schweitz et al., who in 1997 reported a novel method for preparing monolithic MIP capillaries designed for CEC [31]. The initial step in this procedure was derivatization of the fused-silica capillary wall with methacryloxypropyl trimethoxysilane to provide anchoring points to which the MIP could bond. A solution of MAA, TRIM, AIBN and template was prepared in toluene and flushed through the capillary with a syringe. Polymerization was conducted at −20°C for 80 min. The resultant MIP was sufficiently permeable to permit flushing with mobile phase at relatively low pressures. Electron microscopy of the capillaries showed a monolithic polymer consisting of aggregates of micrometer-sized particles and characterized by large interconnected pores on the order of 1–20 μm in diameter. These ‘superpores’ were primarily responsible for the observed permeability to flow. The monoliths performed well chromatographically; the *R* and *S* enantiomers of propranolol were resolved in less than 2 min. An advantage to this approach is that the highly porous monoliths were produced with toluene as the porogen, thereby avoiding the adverse effects (reduced selectivity) typically associated with protic solvents. Under these conditions, the authors found that polymerization time was a critical element in obtaining MIPs with the desired superporous structure. In a subsequent paper, this group reported that incorporating 1–25%

(v/v) isooctane into the porogen provided an alternative mechanism by which these pores could be obtained [32].

3. Applications

To date, most applications of MIP-CEC have been in the area of chiral separations [23–25,31,32]. In one such application, Tan and Remcho [23] employed open tubular MIP-CEC to obtain baseline separations of D- and L-dansyl phenylalanine. Using 25-μm I.D. capillaries, enantiomeric separations were achieved in OT-LC and OT-CEC modes. The OT-LC runs were conducted in a nonaqeuous eluent consisting of 99.5% acetonitrile and 0.5% acetic acid. The eluent conditions were modified slightly for the CEC separations by incorporating an aqueous phosphate buffer in order to generate sufficient EOF. With a mobile phase consisting of 90% acetonitrile and 10% 10 m*M* phosphate buffer pH=7.0, a reasonable EOF velocity was obtained (1.14 mm/s at a field strength of 300 V/cm). As shown in Fig. 4, the dansylphenylalanine isomers were baseline resolved. As predicted, efficiency for the non-imprinted D-isomer was consistently higher in CEC mode relative to OT-LC under similar conditions.

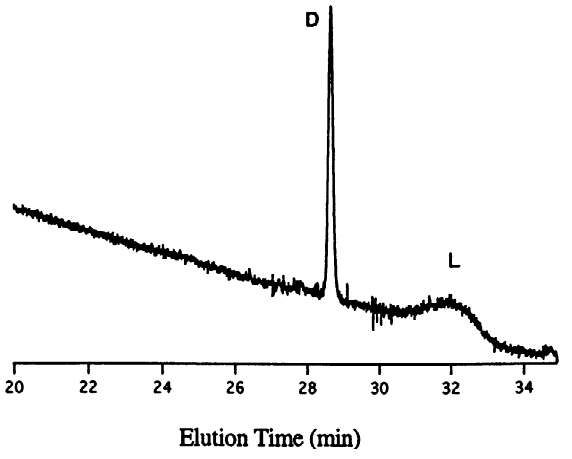

Fig. 4. Separation of D- and L-dansylphenylalanine by open tubular MIP-CEC. Conditions: capillary I.D.: 25 μm; length: 100 cm; mobile phase: 10:1 (v/v) acetonitrile: 10 m*M* phosphate buffer pH 7.0; injection: 40 mbar, 3 s; detection: UV 280 nm (from Ref. [23] with permission).

Rapid enantiomeric separations of the β-adrenergic agonists metoprolol and propranolol have been achieved by CEC on superporous monolithic MIPs [31]. The separations were performed in 75-μm I.D. capillaries with acetonitrile: acetate buffer pH 3.0 (80:20) v/v as the mobile phase. Metoprolol enantiomers were resolved in less than 8 min at a relatively modest field strength of 143 V/cm. Using a higher applied field of 857 V/cm, *R* and *S*-propranolol were resolved in less than 120 s. This separation is shown in Fig. 5. Each of these separations was conducted at elevated temperature (60°C), a technique often employed in MIP chromatography to improve peak symmetry and facilitate more rapid separations. The monolithic capillaries were reported to be quite stable; capillaries stored for 12 weeks exhibited no loss of selectivity. Under the conditions employed in this study, relatively modest EOF was observed. The mobility of mesityl oxide, a neutral marker was calculated as 7.79×10^{-9} m^2/Vs. The analytes, which are amines, evidently were transported through the capillary predominately by electrophoresis.

An interesting application of MIP-based chromatographic sorbents is their use as screening tools for combinatorial libraries [33,34]. The synthesis of large libraries of compounds by combinatorial techniques is becoming an increasingly important tool in the discovery of novel pharmaceutical agents. In combinatorial chemistry, vast libraries of compounds are synthesized using a given skeleton structure as a

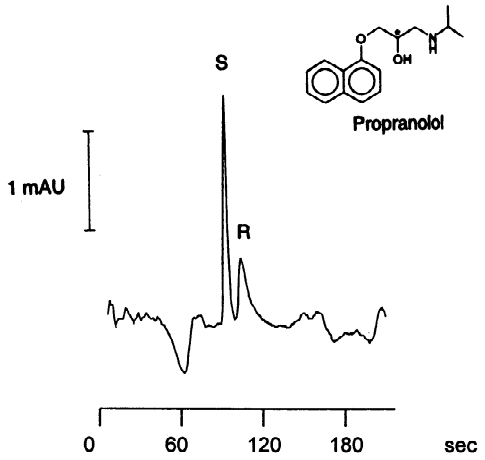

Fig. 5. CEC separation of propranolol enantiomers obtained on a monolithic MIP capillary (from Ref. [31] with permission).

template. The group of candidate activators constituting the library must then be screened against a target receptor (or receptors) to determine which interact most favorably and therefore might be worthy of further study as a potential new drug entity.

The typical compounds generated through current combinatorial methods are small organic molecules with one ionizable functionality, at least one aromatic group and moderate polarity [35]. These types of molecules are amenable to current molecular imprinting techniques. Thus, one might envision utilizing MIPs in the screening process. The general approach would be to select a known, potent activator of the target receptor for use as the imprint molecule. In the idealized case, the recognition cavities within the MIP could serve as mimics of target receptor's binding site. Through screening of the library with the MIP, the relative affinities of the candidates could be assessed. The advantages of MIPs in general would make them attractive for use in this application. The good physical and chemical stability, low cost and ease of preparation of MIPs could in certain instances provide practical alternatives to natural biological selectors in early screening steps, though bioassay would remain an important step in the confirmation of activity.

A new study was recently undertaken in our laboratory to investigate the feasibility of MIPs as screening tools for combinatorial libraries using CEC and capillary HPLC [26]. A group of structurally similar tricylic antidepressant drugs (TCAs) was chosen to simulate a small combinatorial library. MIPs were prepared via bulk polymerization using one of the tricyclics, nortryptiline, as the template molecule. Imprinted polymers were synthesized in bulk using EDMA as cross-linker, MAA and styrene as functional monomers and toluene as porogen. Polymerization was initiated by UV irradiation at $-10^{\circ}C$. The resultant material was ground and sieved to a particle size of <30 μm. After multiple sedimentation steps in acetone to remove fines, the MIP particles were slurry packed into fused-silica capillaries using a syringe pump. For CEC, 100-μm I.D. capillaries were employed.

The optimized eluent consisted of acetonitrile: 10 m*M* sodium acetate pH=3.0 (98:2) to which was added 0.02% v/v trifluoroacetic acid and 0.015% v/v triethylamine. As illustrated in Fig. 6, analysis of the TCA library under these conditions revealed selective retention of the template molecule, nortriptyline. With an applied field strength of 900 V/cm, nortriptyline was separated from the other TCAs in the library, which eluted as single peak, in less than 2.5 min. It is interesting to note the structural similarities between the template and other compounds in the library. The selectivity of the MIP is particularly evident upon inspection of the structure of amitripyline, which differs from the template only by the presence of a methyl group on the pendant amine, and yet was not recognized by the MIP. Such rapid and selective separations illustrate the potential power of MIP-CEC. Further findings from this research effort will be shared in the near future.

4. Conclusion

The advantages of MIPs, namely cost effectiveness, physical and chemical stability, ease of preparation and ability to predetermine selectivity, make them particularly attractive for use as chromatographic stationary phases. Although numerous articles have been published and many successful separations obtained with MIP stationary phases in HPLC, the major limitation has been poor efficiency and peak shape. CEC continues to enjoy growth in the separations community owing in a large part to its efficiency advantage over capillary HPLC. Utilizing molecular imprinted polymers in CEC provides a mechanism by which a major disadvantage of one technique can, in principle, be addressed by the major advantage of the other: the high efficiency of CEC may to a certain extent address the problem of low efficiency often encountered with MIP sorbents. One of the advantages of combining the high selectivity of MIPs with the enhanced flow dynamics of CEC is the potential for rapid chiral separations. Although vast improvements in efficiency have not yet been realized, MIP-CEC shows much promise. The future of the technique will likely see continued application to chiral separations, especially for compounds that are difficult or impossible to separate on conventional chiral stationary phases. Problem areas in MIP-CEC that will require continued research effort include (1) the development of sorbents that

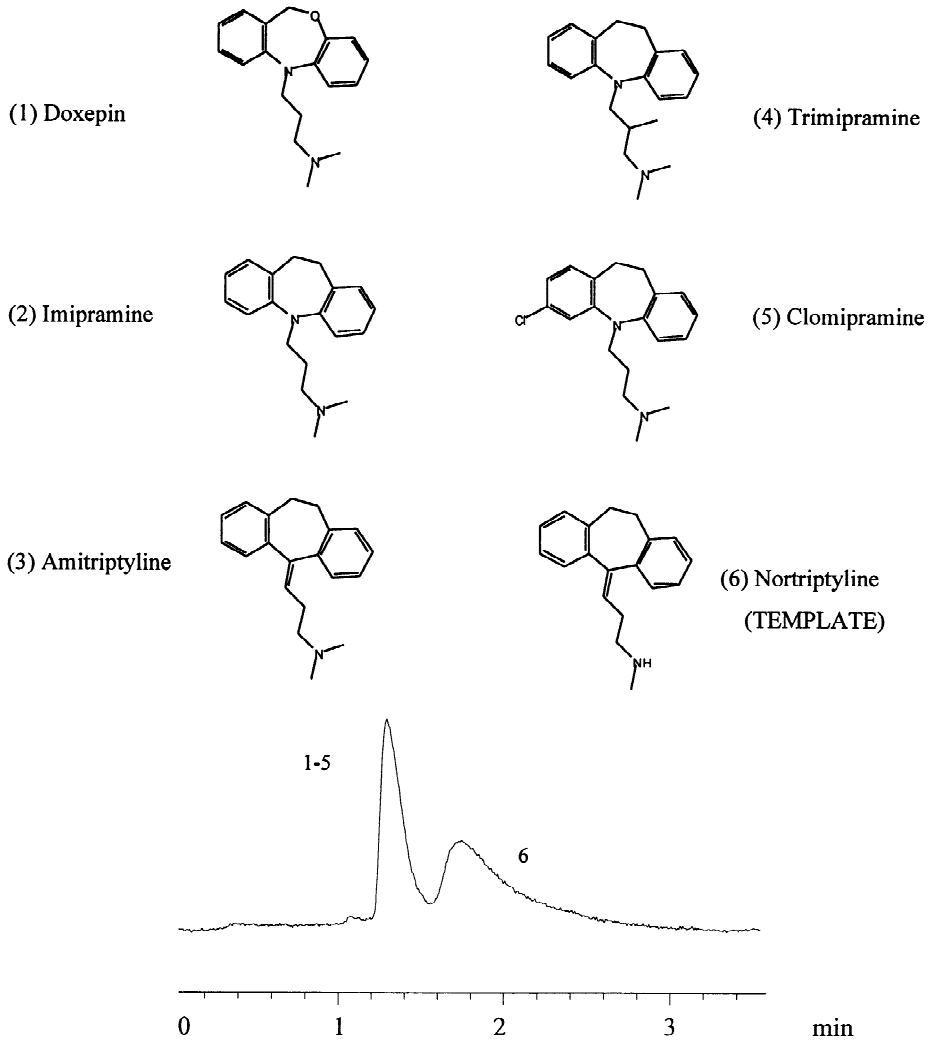

Fig. 6. MIP-CEC separation of a simulated combinatorial library consisting of several tricyclic antidepressants. Conditions: capillary I.D. 100 μm; Ltot: 33 cm; Lbed: 22.5 cm; eluent:acetonitrile: 10 m*M* Na acetate pH 3.0 (98:2) with 0.02% trifluoracetic acid and 0.015% triethylamine (v/v); voltage +30 kV constant; injection: +2 kV, 2 s; column temperature: 50°C.

display both excellent selectivity and good support for electroosmotic flow; (2) more quantitative approaches to the assessment of MIP recognition processes; and (3) the development of methods that produce more uniform imprint sites.

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