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Journal of Chromatography A, 1044 (2004) 53–66
JOURNAL OF CHROMATOGRAPHY A
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Review

Approaches to imprinted stationary phases for affinity capillary electrochromatography

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Abstract

The combination of two highly attractive technologies in analytical chemistry, namely molecular imprinting and capillary electrochromatography, is reviewed. Molecular imprinting represents an approach to incorporate affinity binding sites for one or a class of target molecules in network polymers whereas capillary electrochromatography is a technique that profits from the high separation efficiency of electrodriven separations and the high selectivity and capacity available with liquid chromatography. The review discusses and compares the various approaches taken, to combine these concepts aiming at robust, reproducible and easily available capillary-based affinity separation media.

Keywords: Reviews; Affinity capillary electrochromatography; Electrochromatography; Molecular imprinting; Stationary phases, electrochromatography

Contents

1. Introduction

The imprinting of polymers with templates to yield molecularly imprinted polymers (MIPs) with memory for the original template constitutes an extremely attractive and general approach to molecular recognition (Fig. 1) [1]. The template serves here as a structure directing agent for the formation of template complementary binding sites. Thus, they can be programmed to recognize a large variety of target structures with antibody-like affinities and selectivities. Due mainly to their robustness and ease of preparation, the materials are being studied in widely different contexts encompassing solid-phase extractions (SPE) [2,3], sensors [4], enantiomer separations [5], drug discovery [6], drug delivery [7], and catalysis [8]. Among these, most activities have so far focused on the use of MIPs as tools in analytical chemistry, notably as stationary phases in SPE to achieve highly selective separations of one or a group of target molecules from complex mixtures. Miniaturized separation formats are in this context gaining in importance.

During the last decade, efforts to adapt molecular imprinting to capillary electrochromatography (CEC) has resulted in several innovative contributions to CEC stationary phase

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doi:10.1016/j.chroma.2004.05.055

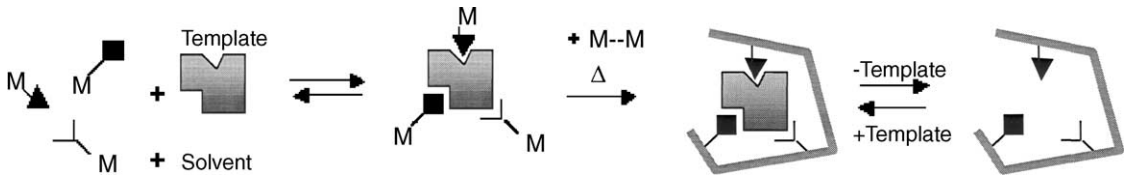

Fig. 1. Concept of molecular imprinting.

technology (Table 1). At the beginning of 1997, while CEC was undergoing a rapid phase of investigation and the number of reports was exponentially growing, the interest in CEC from molecular imprinting (MI) researchers suddenly burst, and further expanded in the following years (Fig. 2).

Molecular imprinting technology has a high potential, in that it can offer robust and easy to prepare materials endowed with predetermined selectivity for a given compound, its analogues or for a single enantiomer. It is, therefore, particularly suitable for routine analysis, provided that adequate validation of its performance is carried out. The main attractive features of CEC are the miniaturized format, the intrinsic higher efficiency and the intrinsic absence of back pressure of an electrically-driven system, allowing the use of capillary packings made up of very small particles. All these features fit well with the limitations observed for the MIP material as chromatographic stationary phase, namely slow mass transfer kinetics, packing irregularities and costs given by expensive or otherwise difficult to find templates or monomers. Moreover, most MIPs are based on the same type of polymer matrices commonly used in the in situ preparation of flow through monolithic stationary phases for capillary or microfluidic systems, and a merge of these technologies therefore seems very attractive.

However, the initial efforts towards combining molecular imprinting with CEC faced several problems, i.e. the technical difficulties of packing irregular MIP particles, of making frits other than by direct burning, of regenerating the background electrolyte in a column made of dense polymer, together with the well known drawbacks of CEC, like capillary fragility and bubble formation. Nevertheless, a series of recent reports suggests that im-

Table 1
Summary of studies in which MIPs have been applied in different CEC formats

MIP–CEC format	Template molecule	Monomers used	Ref.
In situ precipitation polymerization	Benzamidine pentamidine	MAA/EDMA	[9]
Monolithic “flow through” MIPs	(L)-Phenylalanine anilide	MAA, 2-VPy/EDMA	[11]
	(L)-Phenylalanine anilide	MAA, 2-VPy/EDMA	[10]
	(*R*)-Propranolol	MAA/TRIM	[12]
	(*S*)-Metoprolol		
	(*R*)-Propranolol	MAA/TRIM	[14]
	(*S*)-Ropivacaine	MAA, 2-VPy/TRIM, PETRA, PETEA, EDMA	[13]
	(*S*)-Propranolol	MAA/TRIM	[15]
	(*S*)-Propranolol, (*S*)-Metoprolol, (*S*)-Atenolol	MAA, MMA, BMA, EPMA/TRIM	[16]
	4-Aminopyridine	MAA/EGDMA	[17]
Packed MIPs polymer particles	(L)-Phenylalanine anilide, (L)-Phe	MAA/EDMA	[18]
	(L)-Phe, (L)-phenylalanine anilide, (L)-Try, Dns-(L)-Leu	MAA/EDMA	[19]
	(L)-form of Dansylaminoacids	MAA, 2-VPy/EDMA	[20]
Packed MIPs composite silica beads	(L)-Phenylalanine anilide	MAA/EDMA	[21]
	(L)-Phenylalanine anilide	MAA/EDMA	[22]
Entrapped MIPs	(L)-Phenylalanine anilide	MAA/EDMA	[23]
	(D,L)-Dns–Phe	n.r.[a]	[24]
Open tubular MIP–CEC	(*S*)-Phenylpropionic acid	*trans*-3-(3-Pyridil)-acrylic acid/EDMA,DVB	[25]
	Dns-(L)-Phe	MAA, 2Vpy/EDMA, TRIM	[26]
	(*S*)-Propranolol	MAA/TRIM	[27]
	9-Ethyladenine	MAA/EDMA	[28]
MIP as additive in the BGE			
TFT	(*S*)-Propranolol	*N*-Acryloyl-alanine/EDMA	[29]
PFT	(*S*)-Propranolol	MAA/TRIM	[30]
	(*S*)-Propranolol	BMA, MMA/TRIM, PETEA	[32]
	(+)-Ephedrine hydrochloride	MAA/TRIM	[31]
	(*S*)-Propranolol	MAA/TRIM	[33]
	(*S*)-Ropivacaine, (*S*)-propranolol	MAA/TRIM	[34]

[a] Not reported.

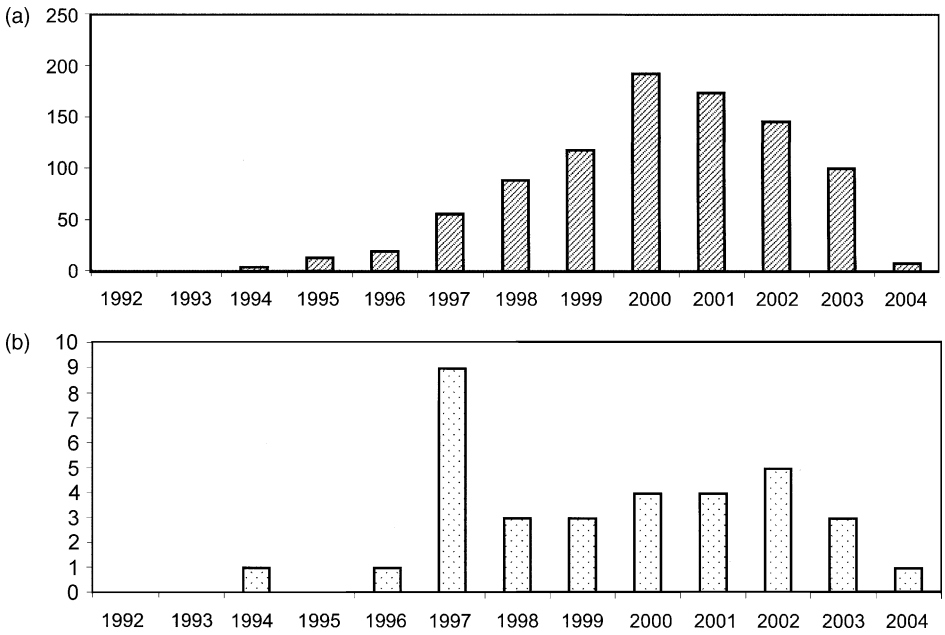

Fig. 2. (a) Number of papers and review articles concerning application of CEC based on PubMed and Science Citation Index search. (Year 2004 is covered to 29 April). (b) Number of papers and review articles concerning application of MIP–CEC based on PubMed and Science Citation Index search. (Year 2004 is covered to 29 April)

portant steps have been taken towards overcoming these difficulties.

The work in this area will be critically reviewed here, starting from the pioneering paper appearing in 1994 [9], through the advent of MIP monoliths [10–17], the evolution of slurry-packed MIP particles [18–22], and of gel-entrapped MIP particles [23,24], followed by MIP films for open tubular capillaries [25–28]. The use of MIP micro- and nano-particles as additives in the BGE [29–34], which is more a pseudostationary phase for electrokinetic capillary chromatography rather than a proper CEC format, will also be included.

The scientific production in this field has so far focused on model systems for chiral separations based on the non-covalent imprinting technique. It has to be mentioned that the frequent use of a model compound as a template reflects the general attention to the testing and the optimization of a novel CEC format rather than to proper applications. However, once formats that meet the requirement in terms of reproducibility, robustness, selectivity and ease of fabrication have been found, applications as stationary phases for capillary chromatography or CEC and for capillary based SPE are foreseen.

2. MIP formats in CEC

The main MIP–CEC formats and their relative benefits and limitations have been listed in Table 2. These will now be discussed in more detail, based on associated reports from the literature.

2.1. Monolithic or particulate in situ prepared MIPs

Monolithic columns are, by definition, continuous porous rods of stationary phase inside the column. [35]. They offer important advantages, particularly for MIP–CEC applications, due to the easy in situ preparation and the absence of retaining frits. However, they rely on the presence of large pores to provide convective flow through the stationary phase. In the case of MIPs, these pores must be generated simultaneously with the generation of the molecular scale binding sites, which constitutes the main limitation of this approach [36].

The main parameters controlling the pore size distribution are temperature, crosslinking level and the nature and content of porogenic solvent. Thus, in thermally initiated polymerizations, large pores are promoted by low polymerization temperatures, lower crosslinking levels and the use of poor solvents for the growing polymer chains. Successful formation of stable imprinted sites, on the other hand, is typically promoted by low polymerization temperatures, high crosslinking levels and aprotic poorly polar porogens [1]. As will be discussed below for some systems, these respective criteria coincide leading to facile in situ preparation of capillary based affinity media.

By following the method proposed by Svec and Fréchet in 1992 [37] to synthesize macroporous polymers, Matsui et al. [38] and Sellergren [39] presented the first example of in situ prepared monolithic [38] or particulate [39] MIPs for HPLC applications. In the latter case MAA, EDMA and AIBN dissolved in isopropanol and water were used to imprint the AIDS drug pentamidine. Isopropanol and water were

Table 2
Characteristics related to capillary preparation for the different approaches to imprinted capillaries for CEC

Format	Benefits	Limitations
Monolithic or particulate in situ prepared MIPs	Simple in situ preparation	Extensive optimization required for each new template system
	No retaining frits required	Preparation may be incompatible with imprinting conditions
		Monolith shrinkage
	Solvent and electrolyte exchange for "superpore monoliths"	PTFE sleeve necessary for thermally initiated monoliths
	Short end injection reported	Bubble sensitive system
Capillaries packed with ground MIPs	MIP prepared and optimized independently from the capillary preparation	More time consuming due to the two step procedure
		Poor control of particle size and distribution
		Packing and frit fabrication
		PTFE sleeve necessary
		Poor efficiency
Capillaries packed with composite silica beads	MIP prepared and optimized independently from the capillary preparation	More time consuming due to the two step procedure
	Thin films with superior mass transfer properties available	Packing and frit fabrication
	Known silica based technology for packing and frit fabrication	
	Proven column and run reproducibility	
Entrapped MIPs	MIP prepared and optimized independently from the capillary preparation	More time consuming due to the two step procedure
	No retaining frits required	Little is known concerning column properties and if the approach is general
	Mechanical robustness	Low sensitivity
		Poor control over porous properties
Open tubular MIP–CEC	Simple in situ preparation	Low sample load capacity
	No retaining frits required	General low plates for the template peak
	Clogging and bubble issues avoided	
MIPs as additive in the BGE	Simple capillary regeneration and electrolyte exchange	Requires access to low dispersity particles in the nanometer size range
	No retaining frits required	Demanding optimization of analytical parameters for the partial filling technique
	Selectivity tuning by mixing MIPs	Capillary initial conditioning and inter-run washings to be addressed for reproducibility
	Packing not required	Polymer and analyte migration velocity and direction limitations
	Short end injection for ultrafast analysis feasible	Polymer-polymer interactions with commercially available coated capillaries

needed in order to solubilize the monomer-template pair and subsequently to produce particle agglomerates with sufficient permeability to the flow. The same monomer–porogen system was also used in the first example reported in the literature to generate imprinted methacrylate polymer for CEC (Fig. 3) [9]. Untreated fused silica capillaries were filled with a mixture of monomers, porogen and initiator and the polymerization was initiated thermally. This simple procedure resulted in packings exhibiting surprisingly high separation efficiencies. The electrolyte could be mechanically pumped through the capillaries, allowing rapid phase changes and micro-chromatographic possibilities. Due to the weak cation exchange properties of the carboxyl groups containing phase, CEC of basic analytes with organic solvent-buffer mixtures as electrolytes was successful, resulting in plate numbers (N) for D,L-phenylalanine anilide of 150,000 m^{-1} and for the nucleoside derivative tri-*O*-acetyladenosine of 53,000 m^{-1} (Fig. 4).

The template pentamidine was strongly and selectively retained on the capillary containing the imprinted polymer. Similar to the corresponding HPLC evaluation, this effect was strongly pH dependent. At pH 4, the retention times for benzamidine and pentamidine were 7.2 and more than 50 min, respectively, on a pentamidine-selective capillary, while they were only 6.5 and 7.2 min on a reference capillary imprinted with benzamidine. The pentamidine selective capillary thus exhibited a pronounced selectivity for pentamidine that could be controlled by the electrolyte pH (Fig. 5). The use of protic solvents is, however, undesirable in the preparation of the majority of non-covalent

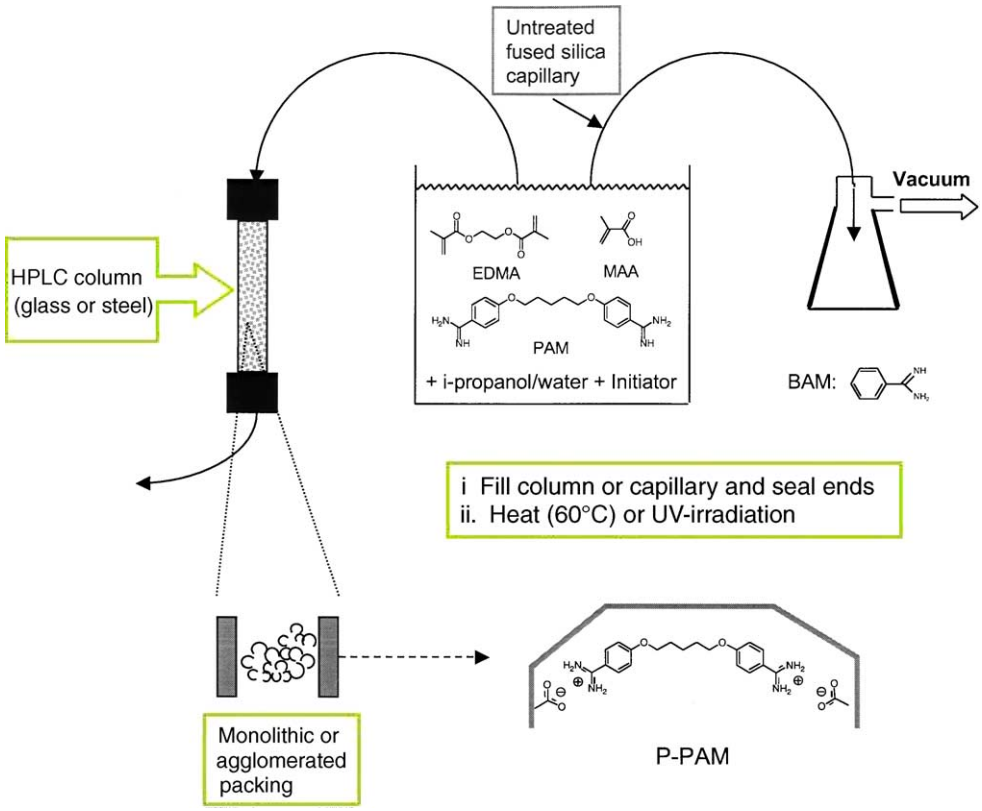

Fig. 3. In situ preparation of molecularly imprinted polymeric packings for liquid chromatography or capillary electrochromatography. Example of HPLC packings imprinted with pentamidine (PAM): PAM (0.125 mmol) in the free base form was dissolved in 2-propanol (2.8 ml) and EDMA (12 mmol). Addition of MAA (0.5 mmol) caused formation of a precipitate, which dissolved after addition of water (1.3 ml). Initiator (AIBN, 12 mg) in 2-propanol (0.5 ml) was added, the solution purged with nitrogen, and heated to 40 °C to achieve homogenization. The solution was then transferred under vacuum to fused silica capillaries. The capillary ends were left immersed in Eppendorf tubes containing the remaining prepolymerization mixture and the assembly left in an oven at 60 °C for 24 h.

imprinted polymers due to their destabilizing effect on the monomer–template interactions. The scope of the method was therefore widened by the introduction of monolith preparation procedures based on the use of aprotic low polar solvents [10–12]. In the reports by Lin et al., a monolithic polymer selective for L-phenylanine anilide was prepared. In order to covalently anchor the polymer to the capillary, the inner wall was derivatized with vinyl groups. The polymerization mixture, composed of methacrylic acid (MAA), 2-vinylpyridine (2-Vpy), ethylene dimethacrylate (EDMA), and of a free radical initiator azo-*N*,*N*′-bisisobutyronitrile (AIBN) in ammonium acetate and chloroform, was then flushed through the capillary and the polymerization allowed to proceed at 60° for 24 h. It was not disclosed how the porogen was replaced with the electrolyte buffer. This question is motivated since these polymerization conditions should lead to monolithic materials that lack flow through pores and appear gel-like in the dry state. Nevertheless, the resulting capillaries were reported to give baseline separations of the enantiomers of the corresponding free amino acid phenylalanine.

More attention to the pore system connectivity was paid by Schweitz et al. [12–14], who introduced flow-through pores into the polymeric network by prematurely terminating the polymerization [12]. After the activation of the inner surface of the silica capillary with (methacryloyloxy)propyltrimethoxysilane, the polymerization mixture, composed of MAA, trimethylolpropane trimethacrylate (TRIM), (*R*)-propranolol or ((*S*)-metoprolol) in toluene, was pumped into a UV-transparent fused silica capillary. The ends of the capillary were sealed and the UV-initiated polymerization carried out at −20 °C. The low temperature was motivated by the higher stability of the template–monomer complexes observed in the pre-polymerization mixture at this temperature [40]. The reaction was stopped just after 80 min and the unreacted monomers were removed by pressurized washing resulting in stationary phases, as viewed from scanning electron micrographs, consisting of micrometer-size globular polymer aggregates separated by pores with a diameter between 1 and 20 μm. Following hydrodynamic replacement with the electrolyte to be used in the electrochromatographic separation, good enantioseparations of propranolol and metoprolol (R_s(propranolol) = 1.26 and R_s(metoprolol) = 1.17) were obtained allowing also quantitative analysis of nonracemic mixtures (Fig. 6).

It can be assumed that the reproducibility of this procedure suffers from the fact that the polymerization is prematurely interrupted. The difficulties in precisely controlling

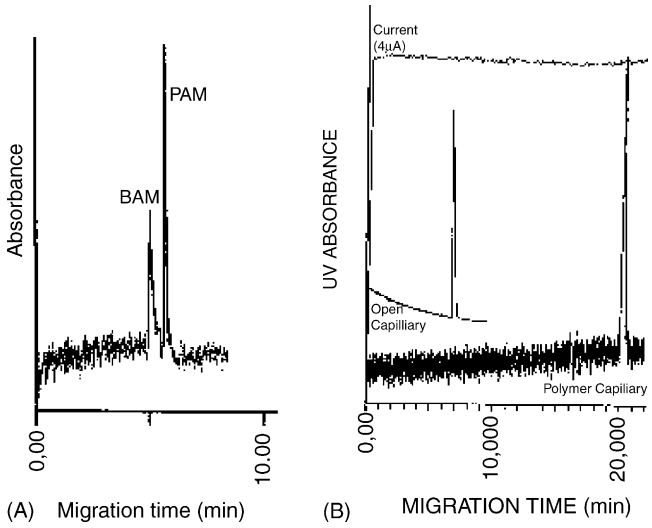

Fig. 4. (A) Electrochromatograms of pentamidine (PAM) and benzamidine (BAM), using a capillary containing in situ prepared packing imprinted with PAM (P-PAM). From [9] with permission. Running buffer: acetonitrile–0.5 M potassium phosphate, pH 2 (70:30 (v/v)). Injection: 5 kV, 3 s. Applied voltage, 5 kV; current, 4.2–4.5 μA; detection, BAM: 254 nm, PAM: 280 nm. (B) Electropherogram of D,L-phenylalanine anilide (D,L-PA) injected (0.33 mg/ml, 6 kV, 3 s) on a capillary (L = 25 cm, i.d. = 100 μm) containing in situ prepared packing imprinted with L-PA. From [9] with permission. Running buffer: ACN–[0.5 M potassium phosphate, pH 5] (7:3 (v/v)). Applied voltage, 6 kV; current, 3.8 μA; detection, 254 nm. Inlay: The peak in an identical run using an open deactivated capillary has been indicated.

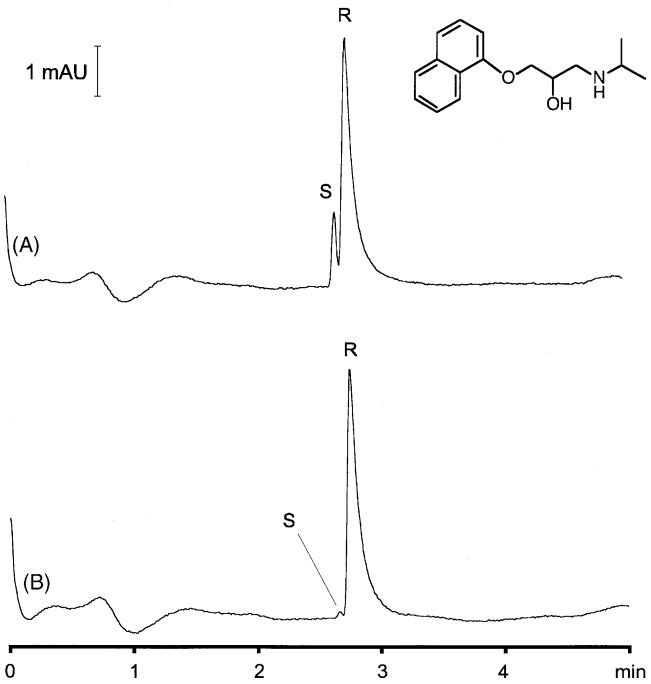

Fig. 6. CEC resolutions of non-racemic mixtures of propranolol on a capillary column packing prepared using (*R*)-propranolol as template. From [12] with permission. (A) 9:1 mixture of (*R*)- and (*S*)-propranolol; (B) 99:1 mixture of (*R*)- and (*S*)-propranolol.

the kinetics of free radical polymerizations implies that capillaries prepared identically may still have reached different conversions of the monomers.

In order to address these difficulties, the large pores were instead produced by addition of the poor solvent isooctane to the porogen without premature interruption [13]. Absence of isooctane leads to dense impenetrable polymers, whereas excess isooctane leads to soft, unstable monoliths. The flow-through properties were controlled by hydrodynamic pumping of liquid through the column and by microscope inspection. The best enantiomer selectivity was obtained with a ratio of 9:1 toluene:isooctane, probably due to the presence of both flow through pores and meso- or micro-pores.

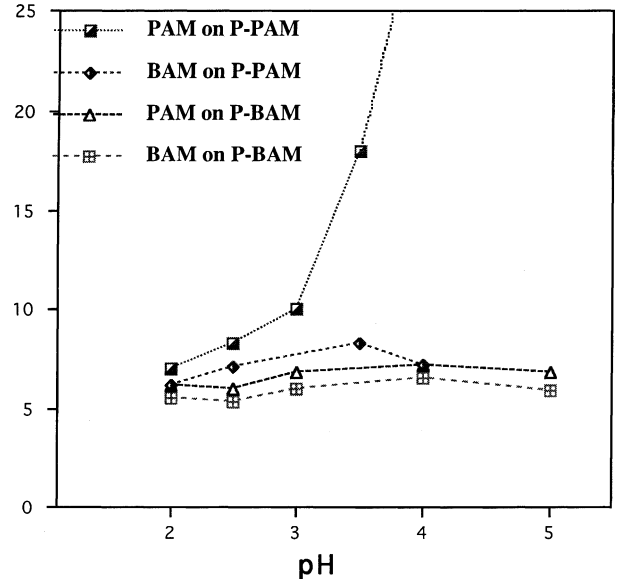

Fig. 5. Plot of the migration time of pentamidine (PAM) and benzamidine (BAM) vs. electrolyte pH using capillaries containing packings imprinted with PAM (P-PAM) and BAM (P-BAM). From [36] with permission. For conditions see Fig. 4A.

Further investigation on the influence of the use of different crosslinkers and of different monomer/template ratios on the permeability and on the recognition properties of the polymers were carried out. The best separation efficiency was obtained for capillaries prepared by using TRIM as crosslinker, whereas the highest normalized separation index ($\Delta t_R/t_R = 0.361$) was observed by using EDMA. High selectivity was promoted by high ratios of MAA to template, whereas resolution was higher for the low MAA/template ratios. Since the above variations also lead to polymers exhibiting different permeabilities, a systematic optimization based on chemometric concepts seems worthwhile.

By decreasing the length of the columns to 8.5 cm (short-end injection) [15], a rapid regeneration and electrolyte exchange was facilitated (back-pressure value down to 1 bar) and enantiomeric separation of *rac*-propranolol was reached in <1 min.

Further modifications of the polymerization protocol were investigated with respect to the monomer composition in the prepolymerization mixture and to selectivity-tuning using multiple templates [16]. In order to tune the hy-

drophobicity of the packing, the hydrophobic comonomer, methyl methacrylate (MMA) or butyl methacrylate (BMA), was included in the polymerization mixture. For capillaries where half of the original content of the functional monomer MAA had been replaced by either MMA or BMA, increased resolution of *rac*-propranolol was obtained. Selectivity tuning was demonstrated using (*S*)-metoprolol and (*S*)-atenolol simultaneously or separately as templates, following investigation of the ability of the MIPs to resolve the enantiomers of metoprolol and atenolol. For both compounds the enantioseparation was increased on the multiply templated column, as compared with the respective singly templated column. As discussed below, an alternative and probably more versatile approach is the use of mixtures of differently imprinted nanoparticles for the same purpose [34].

A MIP-based, thermally-initiated monolithic capillary was more recently described by Yan et al. [17]. By using 4-aminopyridine as template, a 10 cm monolith was prepared by mixing MAA, EGDMA and AIBN in a proper volume of acetonitrile. A high volume ratio solvent to monomer must have been used, as the resulting monolith showed 0.1–5 μm interconnected pores and allowed good permeability. Extensive studies by varying experimental parameters and by using 2-aminopyridine as reference analogue of the template concluded that the mechanism of the separation is based on the interplay among molecular imprinting recognition, ion-exchange, chromatographic retention and electrophoretic migration.

2.2. Packed capillaries

The classical method for preparing MIP-columns for HPLC consists in the synthesis of the polymer in bulk, followed by grinding and sieving to obtain particles with a diameter of 25–35 μm. This approach was also applied for preparing CEC–MIP capillaries [18–20]. Thus Lin et al. prepared polymers imprinted with L-phenylalanine or L-phenylalanine anilide, using the well-established MAA EDMA system with chloroform or acetonitrile-acetic acid as porogens and AIBN as free radical initiator. After grinding and sieving, <10 μm particles were obtained and packed into capillaries of 75 μm i.d.. Frits were prepared from acrylamide gel plugs and a PTFE sleeve was connecting the packed capillary to an empty capillary, providing a detection window. The capillary showed enantioseparation properties, with improved peak shape and resolution obtained in the CEC system when compared with HPLC.

The approach is, however, associated with serious limitations, the most important probably being the need for frits. In particular, the use of acrylamide gel for this purpose may result in non-specific interactions with the analytes, leading to peak tailing and efficiency losses. The irregularly shaped particles and their broad size distribution, obtained by the crushing and sieving technique, further limit the efficiency of the system.

An important advance in this regard was the introduction of MIP–silica composites as new stationary phase materials for CEC [21,22]. These are prepared by grafting thin MIP films from the surface of porous silica beads of a defined size and porosity (Fig. 7) [41]. Notwithstanding the need of frit fabrication by accurate optimization of burning times, there are several benefits of this approach. First, the morphology of the beads and the packing is preset according to the chosen support material and should not be influenced by the imprinting step. Secondly, silica beads are commonly used as packing material in commercially available capillaries for CEC, implying that such capillaries can be prepared with acceptable reproducibility. Thirdly, using

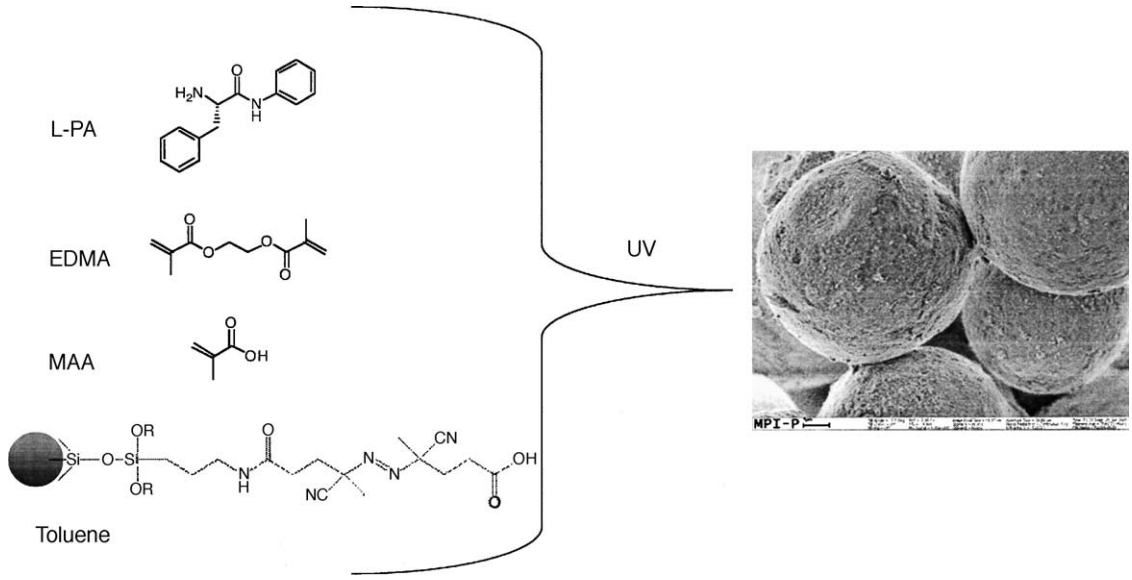

Fig. 7. Preparation of silica supported imprinted polymer films by UV irradiation of initiator-modified porous silica particles suspended in a mixture of template (L-phenylalanine anilide), monomers (MAA and EDMA) and solvent.

the "grafting from" technique, the initiating radicals are generated at the surface of the support, potentially resulting in dense homogenous films with tunable thickness. Thus, HPLC stationary phases could be prepared exhibiting superior mass transfer properties in the racemic resolution of D,L-phenylalanine anilide [41]. In the first reported example, initiator-modified spherical silica particles of different sizes (10 μm (Si 1 0 0), 5–10 μm (Si 1 0 0 0) or 2.8 μm (SiNP) particle diameter) and of different porosities (nonporous or average pore size: 1000 Å (Si 1 0 0 0) or 100 Å (Si 1 0 0)) were suspended in the conventional pre-polymerization mixture composed of MAA, EDMA, AIBN, and the template L-phenylalanine anilide using dichloromethane or toluene as solvents (Fig. 7). Polymerization was subsequently photochemically initiated and allowed to proceed at 15 °C for 24 h. This resulted in a surface-immobilized layer of molecularly imprinted polymer targeted to bind L-phenylalanine anilide.

Fused silica capillaries were packed over a length corresponding to 8 cm, using a pneumatic amplification pump, and the stationary phase thus obtained was tested with respect to its electrochromatographic performance [21,22]. In this step, the presence of silica is an advantage as it allows for frit fabrication by direct burning. The capillaries gave baseline or near baseline resolution of the racemate with column efficiencies and sample load capacities depending on the thickness of the grafted layer and the porosity of the stationary phase (Fig. 8). Interestingly, the packings exhibited different relative efficiencies for the two enantiomers. Whereas the plate number for the template antipode, reflecting the general efficiency of the packing, was highest for the wide pore size packing (Si 1 0 0 0), the highest efficiency for the template was seen for the non-porous packing (SiNP) followed by the narrow pore size packing (Si 1 0 0) (Table 3). Furthermore, the sample load capacity was higher for the narrow pore size (100 Å) materials with a carbon content of 16% than for the wide pore material (1000 Å) with a carbon content of 7%. However, the non-porous packing with a carbon content of 4% exhibited a sample load capacity similar to the narrow pore size packing. The results were discussed in terms of differences in accessibility to the binding sites of the packings and of the mechanism of electroosmotic flow (EOF) generation. These CEC capillaries exhibited similar enantioselectivity, comparable efficiency and high robustness when compared with previously reported molecularly imprinted capillary-based separation media. Perhaps more important was the positive result of the partial validation showing good intra-day, inter-day, and inter-capillary reproducibility (Table 4).

By allowing the polymer grafting from the surface of the non-porous particles to proceed to higher conversions, monolithic composite material was obtained consisting of the nonporous 2.8 μm particles connected via a web-like polymer structure with micrometer-sized interstitial pores (Fig. 9). The grafting step here plays two roles, the introduction of surface functional properties and immobilization of the bed of microparticles in columns or capillaries. It is interesting to note the similarity between this monolithic structure and that of widely used silica monoliths suggesting a new type imprinted monoliths for in situ preparation in columns or capillaries.

2.3. Stationary phases based on entrapped preformed MIP particles

Entrapped MIP columns are prepared by entrapping MIP particles in a polymeric or silica network, so that a homogeneous and stable packed bed is obtained without need for frit fabrication. Early work was reported by Lin et al. [23], where the MIPs were prepared in bulk, and after grinding and sieving, particles (≤5 μm size) were mixed with acrylamide and bisacrylamide in acetonitrile and Tris buffer adjusted to pH 2.5. After filling the capillaries with this solution, a thermal polymerization at 40 °C for 4 h was carried out. This gel was intended to serve as rigid and stable support for polymer particles and also as EOF suppressor. Baseline separation of phenylalanine isomers was achieved by using 10–20% acetonitrile in an aqueous Tris buffer as eluent. Despite efforts to optimize the electrophoretic pa-

Table 3
Comparison of chromatographic data for capillaries packed with imprinted composite particles From [22] with permission

	C%[a]	Acetone	100 μM racemate				200 μM racemate			
		N[b]	N_1	N_2	α[c]	R_s[d]	N_1	N_2	α[c]	R_s[d]
Si 1 0 0 0	4	17750	17728	1774	1.66	0.94	25661	2789	1.07	0.77
	7	17600	12311	1809	1.69	1.25	15999	1690	1.53	0.88
Si 1 0 0	16	11904	6925	2429	1.65	1.68	9972	2493	1.48	1.25
	32	11387	5003	1655	1.67	1.65	5609	1465	1.55	1.26
SiNP	4	11110	2840	2630	4.16	1.55	3393	2500	2.88	1.50

Conditions: CH_3CN–phosphate buffer pH 6.5 (70:30 (v/v)), voltage: −10 kV, injection: acetone, −7 kV, 7 s, λ = 245 nm.

[a] Carbon content obtained by elemental analysis of the composite materials after grafting.

[b] Plate number per meter. $N = 5.54(t_R/W_{1/2})^2$, where $W_{1/2}$ is the peak width at half height.

[c] $\alpha = t_{R2} - t_0/t_{R1} - t_0$, where t_{R2} and t_{R1} are the retention times of the first and the second enantiomer and t_0 is the retention time of acetone, assuming it to be equal to the EOF velocity.

[d] Resolution factor, calculated as $2(t_2 - t_1)/(W_2 + W_1)$.

Table 4
Reproducibility of retention times and enantioselectivity. From [21] with permission

	t_{R1} (min)		t_{R2} (min)		α	
	Mean value	R.S.D. (%)	Mean value	R.S.D. (%)	Mean value	R.S.D. (%)
Intra-day, $n = 9$	1.193	0.89	2.176	1.06	1.542	0.84
Inter-day, $n = 9$[a]	1.909	2.63	2.137	3.56	1.545	0.91
Inter-capillary, $n = 9$[b]	2.174	7.75	2.435	7.13	1.537	0.95

Mobile phase: CH_3CN–sodium phosphate buffer pH 6.5 (70:30 (v/v)). Capillaries packed with particles containing 7% of carbon on the surface. PA racemate concentration: 200 μM.

[a] Three replicates on 3 consecutive days.

[b] Three capillaries (three replicates each).

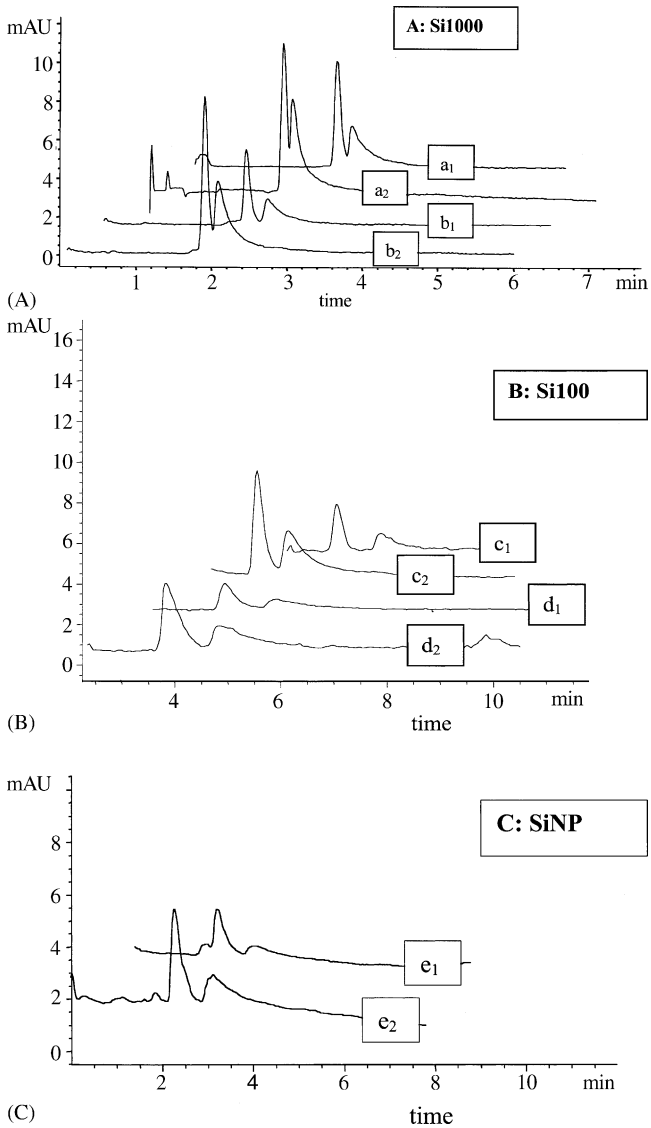

Fig. 8. Chromatograms related to the injection of 100 μM D,L-phenylalanine anilide (a_1, b_1, c_1, d_1, e_1) and 200 μM (a_2, b_2, c_2, d_2, e_2) on capillaries packed with composite particles consisting of porous silica supports containing different amounts of grafted polymer with indicated carbon content. From [22] with permission. (A) a: Si 1000 C4%; b: Si 1000 C7%; (B) c: Si 100 C16%; d: Si 100 C32%, (C) e: SiNP C4%. Conditions: mobile phase: CH_3CN–phosphate buffer 10 mM pH 6.5 (70:30 (v/v)), injection: −7 kV 7 s, voltage: −10 kV, λ: 245 nm.

rameters (mobile phase composition and pH, applied field strength) and the polymer entrapment procedure parameters (acrylamide particle and pore size, MIP concentration), the efficiency of the separation shown was poor. Several drawbacks affect this system, namely low sensitivity, as detection is hampered by the polymer matrix, and absence of hydrodynamic flow, which hampers solvent regeneration.

In 1999, Chirica et al. [24] proposed a silicate-based entrapment method to immobilize Nucleosil ODS particles as well as MIP particles. The polymer particles imprinted for (L)-dansylphenylalanine were packed into a capillary by the classical slurry procedure. A mixture containing 1 ml Kasil 2130 and 2 ml water was then flushed through the capillary. The entrapment was subsequently performed by gradual heating between 40 and 160 °C over several days. A selective retention of (L)-dansylphenylalanine, comparable with that obtained by HPLC, was observed. An advantage of this approach is the increase in the surface charge density which facilitates the formation of EOF independently of the MIP particles. Unfortunately the silicate entrapment medium also contributes to unspecific retention, particularly for compounds containing amine groups.

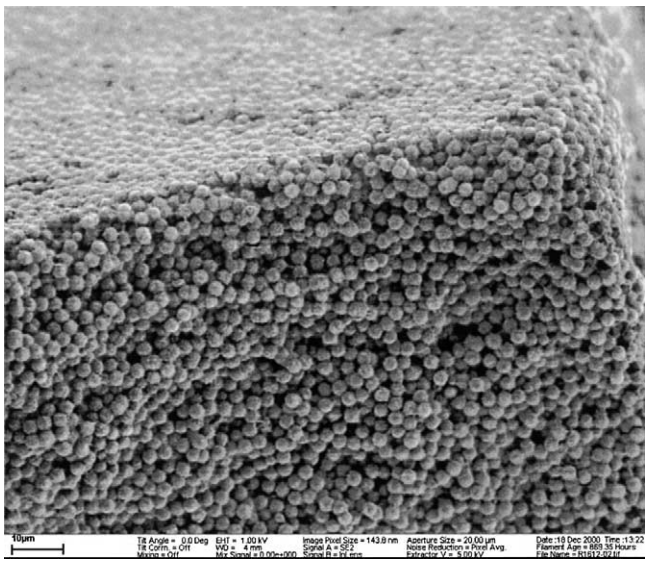

Fig. 9. Scanning electron micrograph of 2.8 μm non-porous SiO_2 particles modified with an initiator after grafting of imprinted polymer to achieve a carbon content of 16% (w/w). From [36] with permission.

2.4. MIPs for open tubular CEC

The open tubular CEC (OT-CEC) format is characterized by the preparation of the stationary phase as a thin layer on the inner wall of the capillary. The preparation of MIP open tubular capillaries offers advantages in that the in situ preparation is simple, and the efficiency in electrochromatographic separations is high. Other intrinsic advantages are the lack of packing induced back pressure which facilitates regeneration and electrolyte exchange, as well as absence of clogging and air bubble formation. An important limitation of this approach, however, is the low sample load capacity due to the low quantity of polymeric material available in the capillary.

The thickness of the MI-capillary coatings should be large enough in order to accommodate the stereoselective binding sites, but small enough to provide a rapid transfer of solutes from the electrolyte to the imprinted sites.

The first example of MIP-OT was reported by Brüggemann et al. in 1997 [25]. After an initial derivatization step of the inner surface of the capillary, the polymerization mixture composed of *trans*-3-(3-pyridyl)-acrylic acid, EDMA or divinylbenzene (DVB), was flushed through a 100 μm i.d. capillary and a thermal polymerization was carried out for 48 h. The remainder of the reaction mixture was then flushed out and the capillary was suitably treated. Seven porogens and two crosslinkers at a concentration between 5 and 20% were investigated. In about 20% of the combinations, a polymer coating of the desired quality was obtained. Successful enantioseparation was inferred from the comparison of the electropherograms obtained for the racemic mixture and the single enantiomers of 2-phenylpropionic acid. The injection of the template enantiomer showed very strong retention as no peaks were detected, possibly also because of the low sensitivity of the system.

Tan et al. produced capillaries of 25 μm i.d. coated with a thin film of polymer imprinted with dansyl-L-phenylalanine [26] by following a procedure previously described by the same authors [42]. After activation of the inner surface, the capillary was filled with a mixture of monomers (MAA and 2-Vpy), crosslinker (EDMA), template (dansyl-L-phenylalanine) in toluene/acetonitrile at different percentages. The capillary was then sealed and the polymerization was carried out at 75 °C. Following this step, vacuum was applied at one end of the capillary and a pressure of 7 bar was applied on the other side in order to replace solvents and to encourage shrinkage of the polymer matrix into a thin film on the capillary wall. For poorly permeable capillaries, this step was instead performed applying a pressure of 100 bar. Capillaries proven impermeable after this treatment were discarded. The success rate in preparing such open tubular columns was about 60%. The ratio of monomer, crosslinker, template and porogen, the incubation time and temperature, were shown to be crucial parameters determining the morphology, selectivity and thickness of the polymer. Pervious capillaries were obtained by employing short synthesis times and MAA (0.2 mmol), 2-VPy (0.2 mmol), EDMA (0.98 mmol), Dansyl-L-Phe (0.25, 0.27, 0.38 mmol) as pre-polymerization mixture in toluene–acetonitrile (ACN) (0.35:0.05, 0.30:0.1 or (0.35:0.05 ml/ml), respectively.

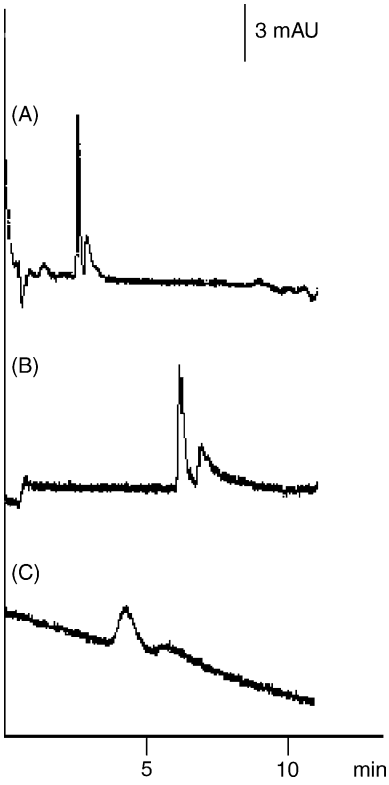

Fig. 10. Electrochromatograms of the enantioseparation of propranolol using MIP coatings synthesized in different solvents. From [27] with permission. (A) Toluene; (B) dichloromethane; (C) acetonitrile. Elution order: (*R*)-propranolol followed by (*S*)-propranolol. Conditions: capillary, 50 μm inner diameter, 35 cm total length, and 26.5 cm effective length; separation, 15 kV; temperature, 60 °C.

By employing a "grafting form" procedure, Schweitz reported on thin homogenous polymer coatings on the capillary wall, resulting in capillaries exhibiting a promising separation performance [27]. The procedure to prepare the coatings was similar to the one developed by Sulitzky et al. [41] to graft thin polymer films from porous silica beads. The pre-polymerization mixture consisted of MAA as functional monomer, TRIM as crosslinker and (*S*)-propranolol as template and three different porogens (toluene, acetonitrile and dichloromethane) were tested. Highest performance was obtained for capillaries prepared using toluene or dichloromethane as porogen, whereas the selectivity was highest for the capillaries prepared using acetonitrile as porogen (Fig. 10). In view of the large thickness of the coatings (0.15–4 μm), this is probably due to the different morphology of the polymers prepared using the different porogens. Very recently, Huang et al. presented capillaries coated with a thermally initiated polymer, specific for 9-ethyladenine [28]. After functionalization of the capillary

wall and careful optimization of polymerization time and polymerization composition, an extensive study was carried out on EOF generation and on the effect of parameters such as buffer pH, buffer concentration, methanol content and voltage. This led to a detailed discussion on the mechanism of complexation; bare fused silica was taken as reference material. This is one of the few papers where migration time reproducibility is addressed and discussed.

2.5. MIPs as additive in the background electrolyte

The use of an additive in the background electrolyte to tune selectivity dates back to the development of micellar electrokinetic chromatography [43] and to the use of cyclodextrins as chiral selectors [44]. Notably this system has also in the past been set up with selectors which elicit a strong detector response, namely macrocyclic antibiotics [45] and proteins [46]. As the selector can be classified as a pseudostationary phase, this field of research is more related to electrokinetic capillary chromatography rather than capillary electrochromatography, and when MIPs were first employed as additives to the background electrolyte [29] the field was already well established. The complete filling of the capillary with a strongly UV absorbing selector has the obvious disadvantage that it leads to a deterioration of the sensitivity, since the analyte needs to be detected on top of a large background signal. This puts an upper limit on the practical selector concentration that can be used. An elegant solution to the problem came with the introduction of the partial filling technique [47,48]. This approach is quite general and can in principle be applied to any kind of selector. This method has been historically defined in two different set-ups: the capillary is either hydrodynamically filled by applying a given pressure for a given time, so to obtain a selector plug of defined length which does not reach the window, or a complete filling is performed prior to analysis, and the selector plug clears the window upon voltage application. In both cases it is evident that analysis conditions have to be carefully chosen, so that the selector migrates toward the injection end and the analyte in the opposite direction. For this reason, a major issue in these systems has always been the suppression of the electroosmotic flow, which can be achieved by using a coated capillary.

Addition of MIP particles to the background electrolyte has many attractive features. It is in principle simple to realize, it avoids time-consuming capillary packing and frit formation and the method development can be fast, as conditions and type of MIP selector can be quickly changed prior to every run. However, it is not overly surprising that the use of MIPs as selectors added to the background electrolyte has been a relatively unexplored area until fairly recently. Despite the simplicity and the attractive features of the set up, the filling techniques also have limitations. The difficulty of filling a tiny capillary with irregular crushed and sieved imprinted particles, together with the drawbacks of the total filling method above mentioned, have hampered any attempts in this direction. The partial filling method requires a demanding optimization of the CE conditions, in order to obtain reproducible and sound data.

Walshe et al. [29] used MIPs as additives in the BGE, by totally filling a 100 μm i.d. capillary with bulk MIPs (20–30 μm particle size) synthesized using *N*-acryloylalanine as a chiral functional monomer and (*S*)-propranolol as template. Despite the good enantioseparation produced, it is difficult to judge whether the separation shown is based on a true imprinting effect or rather on the presence of a chiral monomer in the polymer. The corresponding non-imprinted polymer was too unstable to be tested and the use of an achiral monomer (MAA) failed to provide resolution of the enantiomers. Furthermore, the reported conditions typically result in fluctuating current and in turn in poor run-to-run reproducibility. In particular the use of a 0.05% (w/v) suspension of rapidly sedimenting particles (diameter: 20–30 μm) in pure aqueous electrolyte can be questioned. It is also important to notice that the inter-run rinsing times are usually not reported for this type of applications. This important parameter typically reflects the susceptibility of the system towards capillary clogging, and further reflects data reproducibility and EOF velocity. Finally, the choice of the complete filling method for strongly UV absorbing chemical entities such as MIPs is not advisable, as it inevitably lowers the sensitivity, efficiency and limits the polymer concentration that can be used.

In order to overcome these limitations, De Boer et al. [31] and Schweitz et al. [30] applied the partial filling technique to MIP microspheres prepared by precipitation polymerization. In the work by De Boer at al. [31] 100–200 nm poly(MAA–*co*-TRIM) microspheres imprinted with (+)-ephedrine were used. Despite the weak enantioseparation, low efficiency and long run times (10 min), this first study demonstrated the general feasibility of the approach. EOF suppression was here realized by operating at very low pH (2.5), and any variation from this value compromised the separation. It has to be pointed out that the partial filling method implies some experimental restrictions in that the analyte and the polymer must have opposite apparent mobility, and that EOF suppression obtained by simply operating at low pH results in an irreproducible low EOF value, in limited flexibility of the analytical conditions and in possible adhesion of the polymer to the capillary wall.

In the work by Schweitz et al. [30], stable suspensions of microspheres (0.2–0.5 μm average particle diameter) imprinted with (*S*)-propranolol were used. The microspheres were prepared by following the principles of a precipitation polymerization protocol previously reported [49,50]. They used an essentially identical monomer composition as employed to prepare the flow through monoliths developed by the same group [13,15], but under high dilution conditions, to prevent particle coalescence. In order to suppress the EOF, the capillary wall was derivatized with (methacryloxy)propyltrimethoxysilane and the separations were run at a low operative pH (3.5). As the authors claim a very low

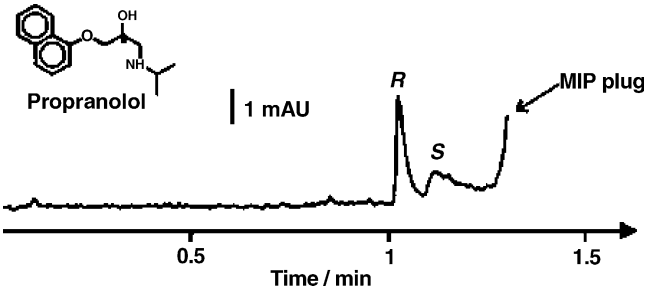

Fig. 11. Electrochromatogram of a typical enantioseparation of propranolol. From [30] with permission. Electrolyte acetonitrile–25 mmol/l phosphoric acid triethanolamine buffer at pH 3.5 (90:10 (v/v)). MIP microparticles were suspended in the electrolyte to a 5 mg/ml dispersion and introduced by applying 50 mbar for 4 s, which corresponds to 11.8 cm of the capillary length. The capillary was of 100 μm i.d., 35 cm total length and 26.5 cm effective length. Sample (25 μmol/l) was injected electrokinetically at 5 kV for 4 s. The separation was performed by applying 15 kV (429 V/cm) at 5 bar overpressure.

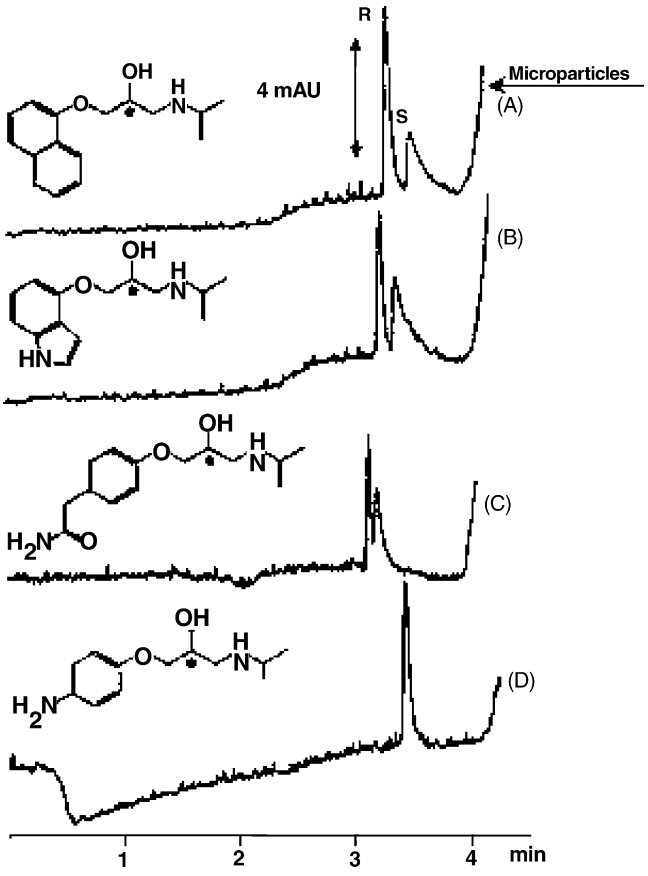

Fig. 12. Cross-reactivity on a capillary partially filled with (*S*)-propranolol imprinted microparticles. From [32] with permission. The particles were injected at 50 mbar for 4 s. The sample was 50 μM of: (A) *rac*-propranolol, (B) *rac*-pindolol, (C) *rac*-atenolol and (D) *rac*-prenalterol, respectively, injected at 3 kV for 3 s. The separation was performed at 50 mbar overpressure, 60 °C at 5 kV. Propranolol and pindolol were detected at 215 nm and atenolol and prenalterol were detected at 195 nm.

operative EOF, it is not clear how the MIP plug, which is deemed to migrate essentially by the EOF, reaches the detector before 1.5 min. As already mentioned above, the EOF suppression when using the partial filling technique is crucial, as it strongly influences the selector plug migration velocity, the plug length and in turn the reproducibility of peak areas and migration times [51], as well as the time window where clear sample detection can be obtained. Nevertheless, good enantioseparations with short run times were seen with a complete resolution of the racemate within 1.5 min (Fig. 11). However, in spite of the small particle size, the mass transfer kinetics was not much improved, as the template peak is still tailing, compared to its enantiomer.

The extremely low consumption of material (4.6 μg of MIP for each run), together with the promising results obtained, prompted the authors to study the same system more thoroughly [32]. Thus, several parameters in the polymer preparation protocol (template to monomer ratio, type and amount of functional monomer, type and amount of crosslinker) and in the electrophoretic run (applied voltage, temperature and electrolyte composition) were optimized. By using these (*S*)-propranolol imprinted microparticles, the presence of "cross-reactivity" was proved, as racemic samples of structural analogues of the template (pindolol and atenolol) were successfully enantioseparated (Fig. 12).

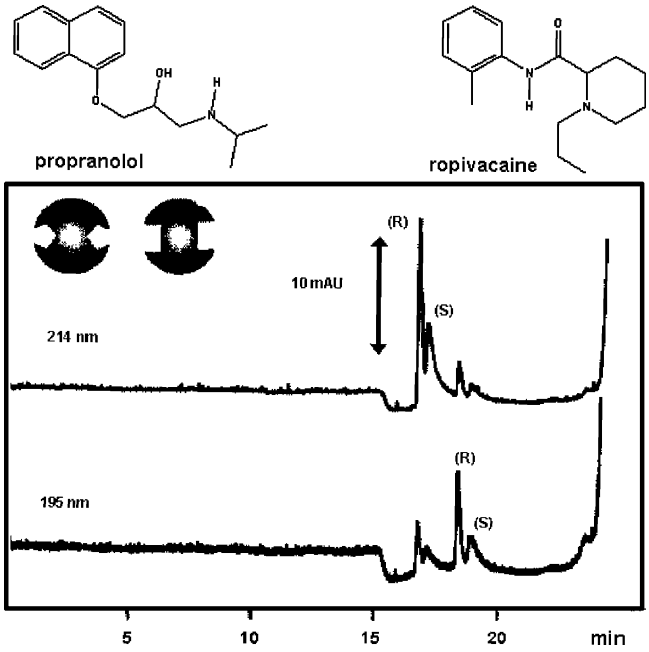

Fig. 13. Separation of ropivacaine and propranolol enantiomers using a MIP plug composed of (*S*)-ropivacaine MIP and (*S*)-propranolol MIP. From [34] with permission. The capillary was 100 cm in total length and 91.5 cm in effective length. The electrolyte contained acetonitrile: 2 M acetic acid adjusted to pH 3 by the addition of triethanolamine (90:10 (v/v)). The separation voltage was 15 kV and the capillary column was thermostated to 60 °C. The two MIPs were injected hydrodynamically at 50 mbar for 6 s each and the sample was composed of 50 mM racemic propranolol (first eluting) and racemic ropivacaine injected electrokinetically at 16 kV for 3 s. Detection was performed at 214 nm (top) and 195 nm (bottom).

The same synthesis protocol was applied in a subsequent work [33], where further optimization led to the separation, in a single run, of the same racemate solution. It has to be mentioned that in this work a fused silica capillary (75 μm i.d.) was used and neither wall derivatization nor pretreatment were reported.

In a recent extension of this approach the selectivity of the MIP selector was tuned either by mixing of two types of MIPs [using (*S*)-propranolol and (*S*)-ropivacaine as templates] or by the inclusion of the two templates in the preparation of a single MIP [34]. In both the cases, a minute amount of material and template is needed. The main gain of the former approach, where two separate MIP suspensions are injected consecutively into the capillary, is the fast and easy optimization of the MIP preparation. Thus, enantioseparation of ropivacaine and propranolol was nicely achieved in a single short run (Fig. 13). The multiply templated MIP showed less satisfactory results.

Both systems are influenced by several electrophoretic parameters including the MIP plug length, the capillary length, the EOF, and also by the particle synthesis parameters such as the type and relative concentrations of the template, monomers and solvent. Notably, a careful and time consuming optimization is often needed to achieve the desired particle size and dispersity. Given the high number of variables, the optimization task when facing a new untested target may be very demanding.

3. Conclusions

The use of robust affinity stationary phases in small columns constitutes an important part of the development of analytical systems combining high throughput, sensitivity and robustness with low sample consumption. As discussed in this review, the affinity in these systems can be provided by MIPs. Due to their tunable selectivity and ease of preparation, the number of MIP-based applications is rapidly increasing. MIP–CEC systems have been recently developed through different approaches, namely as monolithic capillaries prepared by in situ polymerization [10–17], packed capillaries [18–22], open tubular capillaries [25–28], polyacrylamide entrapped MIPs [23,24], and partial filling techniques based on MIP micro- or nano-particles [29–34]. As highlighted above and summarized in Table 2, each of the approaches has benefits and limitations in terms of their general applicability. The in situ preparation of flow through monoliths is technically simple but relies on a fortunate discovery of conditions suitable for generating high affinity imprinted sites and a monolith exhibiting wide pores necessary for convective flow through the bed. The simple use of MIP-particles with a diameter $<10\,\mu m$ to pack CEC capillaries have some serious limitations, the main being the fabrication of frits and the often irregularly shaped particles. Frits prepared from silicate materials, a common practice in CEC [18–20], have been employed with MIP particles, although capillaries prepared in this mode are highly susceptible to bubble formation. Moreover, these previous examples have not addressed the intrinsic problems associated with MIPs as stationary phases, i.e. slow intraparticle mass transfer, binding site heterogeneity and complicated or limited scope preparation protocols.

In this regard, the concept of grafting polymer films from the surface of initiator modified support materials offers an attractive approach to the preparation of capillaries showing high robustness, reproducibility and improved performance [21,22].

Other recent approaches that appear particularly promising in terms of their simplicity and the demonstrated performance are the open tubular CEC systems based on the "grafting from" preparation technique as well as the partial filling techniques [29–34]. The latter approach by passes the need for capillary packings and facilitates tuning of stationary phase selectivity.

Although the efficiency of the MIP–CEC separations reported so far are still poor it should be noted that the use of these packings are not limited to analytical separations. Important applications are also envisaged in the area of sample preparation in miniaturized analytical systems.

Acknowledgements

The authors acknowledge the financial support of the European Union's Human Potential Program under the contract HPRN-CT-2002-00189 (AQUAMIP).

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