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Surface initiated molecularly imprinted polymer films: a new approach in chiral capillary electrochromatography

Milena Quaglia,[a] Ersilia De Lorenzi,*[a] Claudia Sulitzky,[b] Gabriella Massolini[a] and Börje Sellergren*[b]

[a] *Department of Pharmaceutical Chemistry, University of Pavia, Viale Taramelli 12, 27100 Pavia, Italy*
[b] *Institut für Anorganische Chemie und Analytische Chemie, Johannes Gutenberg Universität Mainz, Duesbergweg 10-14, D-55128 Mainz, Germany*

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A new generation of imprinted composite particles was tested as capillary electrochromatography stationary phase. Silica particles characterised by a well defined particle size (10 μm diameter), shape and pore system (1000 Å) were modified with an azoinitiator and subsequently used to graft molecularly imprinted polymers 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. The electroendosmotic flow mobility was evaluated with respect to both the different content of polymer on the silica particle surface and different operating pH values. The dependence of various parameters, namely the analyte concentration, the polymer layer thickness and the pH of the mobile phase on the enantioselectivity was investigated. These CEC capillaries showed enantioselectivity comparable with that showed in LC, and exhibited improved performance in terms of plate number N_1 (~13 000), selectivity α (~1.5), analysis time (< 3 min), inter–intraday and intercapillary reproducibility. We expect this approach to result in a new generation of robust, tailor-made chiral or affinity stationary phases for CEC.

Introduction

Molecular imprinting is a technique devoted to the preparation of highly cross-linked polymers with high affinity for a predetermined class of molecules. The classical way to produce these polymers[1,2] consists in a simple polymerisation route in the presence of a template, template extraction followed by crushing and sieving. The use of these materials as stationary phases for chiral separations is very attractive,[3] particularly because this technique allows a simple preparation of chiral stationary phases with a predetermined enantioselectivity. Unfortunately this application has limits due to the low efficiency of the system, generated by the heterogeneous nature of the binding sites, slow mass transfer and a non-uniform distribution of particle size. The use of MIPs as stationary phases in capillary electrochromatography[4] could increase the efficiency of the system due to the linear flow profile typical of electrophoretic processes, nevertheless without improving the quality of the material in terms of site uniformity and porosity.

MIP-CEC systems have recently been developed through different approaches, namely as monolithic capillaries prepared by an *in situ* polymerisation,[5,6] open tubular capillaries,[7,8] polyacrylamide entrapped MIPs[9,10] and partial filling techniques based on MIP microparticles[11] or nanoparticles.[12] The simple use of MIP-particles with a diameter < 10 μm to pack CEC capillaries has some serious limitations, the main one being fabrication of frits. Frits prepared from silicate materials, a common practice in CEC,[13] have been employed with MIP particles, although capillaries prepared in this mode are highly susceptible to bubble formation. New MIP-silica composite particles, recently proposed as HPLC stationary phases[14] are here envisaged as CEC packings (a preliminary account of this work was presented at the HPCE symposium in Boston, 2001), having the advantages of particle uniformity and of easy frit preparation by direct burning on the stationary phase.

Experimental

Chemicals

All reagents used in this study were analytical reagent grade.

NaH_2PO_4, Na_2HPO_4, CH_3COONa, citric acid and sodium citrate were purchased from Merck (Merck, Darmstadt, Germany); CH_3COOH, acetone, methanol and acetonitrile were from Carlo Erba (Milano, Italy). Depending on the buffering capacity, phosphate buffer was used for experiments at pH 7.5 and 6.5, whereas acetate buffer for experiments at pH 5.5 and 4.5 and citrate for experiments at pH 3.5. Water was deionized by passing through a Direct-Q™ (Millipore) system (Millipore, Bedford, MA, USA). L-Phenylalanine anilide (L-PA) and D-phenylalanine anilide (D-PA) were synthesized at the University of Mainz. Sample preparation was carried out by dissolving known amounts of the analytes in the background electrolyte and each stock solution was diluted at the desired concentration (500, 200, 100 μM).

Synthesis of composite particles

The imprinted composite beads used as packing material are characterised by a uniform size and a homogeneous polymer layer with controllable thickness.[14] The synthesis of these particles, targeted towards the model template L-phenylalanine anilide (L-PA), is shown in Fig. 1. The composition of the grafted polymer was similar to that of the corresponding bulk polymers that were previously extensively investigated as HPLC stationary phases.[15,16] Silica particles with a diameter of 10 μm, a porosity of 1000 Å and different contents of polymer were used in this work. These were modified with the azoinitiator, 4,4′-azo-bis(4-cyano pentanoic acid) (ACPA) in two steps *via* triethoxyaminopropylsilane (APS) to give the initiator-modified support, as shown in Fig. 1. The grafting was then carried out in test tubes containing the initiator-modified particles suspended by a nitrogen stream in a degassed solution of the monomers methacrylic acid (MAA) and ethyleneglycoldimethacrylate (EDMA) and L-PA in toluene. The photochemical initiation was carried out by immersing the test tubes in a thermostatted water bath at 15 °C and placing them near (*ca.* 1–2 cm) a high-pressure mercury vapor lamp used as UV light source. The samples were purged with nitrogen throughout the

polymerization. After polymerisation, the samples were extracted with methanol using a Soxhlet apparatus for 24 h.

Capillary electrochromatography

CEC experiments were performed on a HP 3D system (Hewlett-Packard, Waldbronn, Germany) equipped with a diode array detector, ChemStation software for data processing and an external pressurisation facility (up to 12 bar). Fused silica capillaries (100 µm id, 360 µm od, 36 cm total length) were obtained from Micro Quartz (München, Germany). Using methanol as a slurry solvent, 8 cm of each capillary were packed by the slurry packing technique[17] through a pneumatic amplification pump and a home-made packing device, by flushing $CH_3CN:H_2O$ (30:70) as impact transfer mobile phase at a pressure of 600 bar. The frits were fabricated by burning the stationary phase for different duration times, between 15 and 18 s, depending on the quantity of polymer grafted on the silica particles. A window was created at 8.5 cm from the end of the capillary, and 245 nm was selected as the absorbing wavelength. The capillary was thermostatted at 25 °C during all experiments, and samples were injected electrokinetically by applying −7 kV for 7 s. The operating voltage was set at −10 kV.

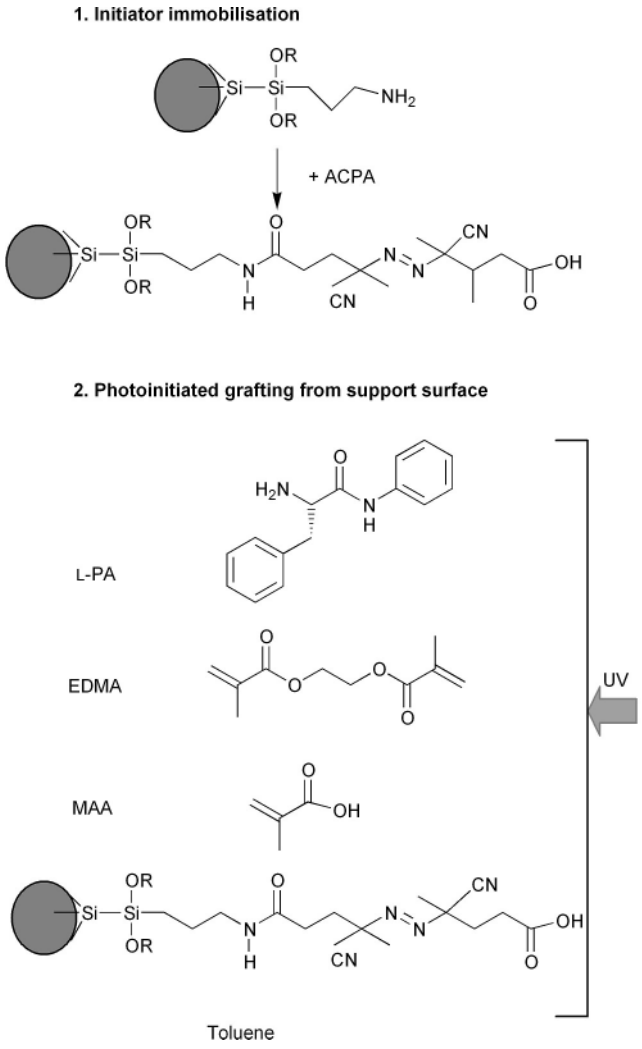

Fig. 1 Procedure for synthesis of L-PA MIP using surface immobilised initiators.[14] See experimental section for details.

Results

Generation of the electroendosmotic flow

The first aim of this study was to investigate the presence, the extent and the origin of the electroendosmotic flow (EOF). The synthetic pathway of the silica-grafted molecularly imprinted polymers implies several consecutive surface modifications (conversion of silanol groups into amino groups, initiator immobilisation and the subsequent polymerisation step) and therefore the generation of EOF given by either the contribution of ionisable groups of the polymer, of unreacted surface-bound amino groups or of unreacted silanol groups needs to be explored. This study was carried out under a voltage of −10 kV, by using CH_3CN: 10 mM sodium phosphate buffer of pH 6.5 (70:30) as background electrolyte and by injecting acetone for 7 s at −7 kV. Under these operating conditions, the calculation of EOF was extended to capillaries packed with particles characterised by a different quantity of polymer (Table 1), evaluated by elemental analysis as percentage of carbon on the surface of the particles (C 7%, C 4%, C 1.6%). The outcome of this investigation indicates that the system generates an EOF mobility value higher than 2.5×10^{-4} cm^2 V^{-1} s^{-1} and that by increasing the quantity of grafted polymer, an increase of the EOF occurs. This latter observation may be tentatively ascribed to the increasing contribution given by the carboxylic acid groups originating from the polymer. In order to gain further insight on the generation of the EOF, this study was also carried out varying the mobile phase pH, and the results are presented in Table 2. The observed reduction of EOF by decreasing the pH value is plausible, due to a lower degree of ionisation of negative charge contributions, namely carboxylic acid and silica groups, and/or an increase in the positive charge contributions originating from the surface amino groups. At pH 3.5 it was not possible to measure the EOF, as even at very long retention times (>40 min) the marker was not detected. The very weak EOF at this pH, expected to be lower than 1×10^{-5} cm^2 V^{-1} s^{-1} is probably indicative of a significant contribution of either

Table 1 Average of measurements in duplicate of EOF mobility values (μ_{EOF}) in capillaries packed with particles characterised by a different quantity of polymer on the surface. EOF marker: acetone, mobile phase: CH_3CN: 10 mM sodium phosphate buffer of pH 6.5 (70:30).

C content on the surface (%)	μ_{EOF}
1.6	$2.574 \times 10^{-4} \pm 0.2 \times 10^{-6}$
4	$2.657 \times 10^{-4} \pm 2.2 \times 10^{-6}$
7	$2.751 \times 10^{-4} \pm 0.2 \times 10^{-6}$

Table 2 Influence of the mobile-phase pH value on the EOF mobility and on the selectivity factor. Mobile phase CH_3CN: 10 mM buffer, 70:30 (v/v), capillaries packed with particles containing 7% of carbon on the surface. Each parameter is the average of duplicate measurements.

		Racemate 100 µM			Racemate 200 µM		
pH	μ_{EOF}	t_{R1}/min	t_{R2}/min	α	t_{R1}/min	t_{R2}/min	α
4.5	$1.662 \times 10^{-4} \pm 1.9 \times 10^{-6}$	1.823 ± 0.022	2.334 ± 0.076	0.589 ± 0.062	1.689 ± 0.015	1.984 ± 0.010	0.786 ± 0.001
5.5	$2.267 \times 10^{-4} \pm 0.6 \times 10^{-6}$	2.562 ± 0.077	3.297 ± 0.131	3.348 ± 0.292	2.484 ± 0.044	2.971 ± 0.082	3.077 ± 0.162
6.5	$2.705 \times 10^{-4} \pm 2.1 \times 10^{-6}$	2.377 ± 0.003	2.750 ± 0.019	1.759 ± 0.051	2.369 ± 0.012	2.623 ± 0.013	1.525 ± 0.011
7.5	$3.433 \times 10^{-4} \pm 1.0 \times 10^{-6}$	1.711 ± 0.009	1.834 ± 0.001	1.552 ± 0.057	1.726 ± 0.009	1.803 ± 0.001	1.326 ± 0.045

the carboxylic groups or amino groups in comparison with the silica groups in the generation of EOF.

Evaluation of the enantioselective properties

The non-grafted bulk polymer corresponding to the polymer here grafted on silica has been previously described as HPLC[15,16] and CEC[9,18,19] stationary phases, showing good enantioselectivity properties. The former system has been thoroughly investigated with respect to its thermodynamic and mass transfer properties,[20–22] molecular recognition properties,[23] polymer structure and morphology.[15,16] A composite material identical to that described here was recently introduced as HPLC column packing and showed a similar enantioselective behaviour but improved efficiency, leading to fast enantiomer resolutions on short columns.[14] In order to test whether this material could be successfully used in CEC as a chiral stationary phase, the capillaries were initially evaluated under conditions that were found to give optimal resolution in the HPLC mode. The results of these evaluations are expressed in terms of enantioselectivity, reflected in the selectivity factor α and in terms of efficiency, reflected in the number of theoretical plates per meter (N m^{-1}). The selectivity factor was calculated as $t_{R2} - t_0/t_{R1} - t_0$ where t_{R1} and t_{R2} are the retention times of the first eluted and of the second eluted enantiomer, respectively, and t_0 is the retention time of acetone, assuming to be equal to the velocity of the EOF. The number of theoretical plates was calculated as 5.54 $(t_R/W_{1/2})^2$. As depicted in Fig. 2a, by increasing the polymer thickness on the surface, the consequent higher number of specific sites produced an increase of the enantioselectivity properties, whereas stronger interactions with the stationary phase induced a progressive lower efficiency when enantioseparation was observed (C 1.6% N m^{-1} = 11 500; C 4%, N_1 m^{-1} = 15 200, N_2 m^{-1} = 1530; C 7%, N_1 m^{-1} = 8790, N_2 m^{-1} = 1470). These results are qualitatively in agreement with the data obtained from the HPLC evaluations,[14] although the optimum was then found at a higher carbon content. No separation was observed for the material containing the lowest carbon content. Assuming a homogeneous coverage of the grafted layer, it is reasonable to suppose that the templated sites will be less selective, due to the layer thickness of only 0.9 nm. Despite the evidence of much better efficiency in the CEC system if compared with the analogous HPLC column recently presented,[14] the number of theoretical plates is still far away from that observed in common silica CEC-systems. This can be justified considering the slow mass-transfer kinetics of the templated binding sites and the heterogeneous distribution of these sites.[21] Fig. 2b shows the effect of different concentrations of racemate injected in the capillary on the enantiomer recognition. A fixed number of recognition sites leads to a nonlinear retention behaviour reflected in an increase in the enantioselectivity with decreasing analyte concentration.

An ion-exchange retention mechanism was previously proposed for the corresponding monolithic polymer evaluated as an HPLC stationary phase using aqueous mobile phases.[16] To understand if the recognition mechanism of the monolithic polymer and of the silica-grafted polymer is the same, the effect of pH on the separation of racemates at two different concentrations was investigated. The results are reported in Table 2. The best enantioseparation was obtained at pH 5.5, confirming the ion exchange mechanism involved in the recognition process reported for the corresponding monolithic material.

One of the main issues in CEC is the reproducibility of results, especially when obtained with home-made columns. For previous MIP based CEC separations, information about reproducibility is hard to find and sometimes not reported. As evident from Table 3, the reproducibility of α values obtained for PA racemate is very good when calculated within the same day, interday and also notably between different capillaries.

For a further optimisation of the CEC system, ongoing studies include the influence of the mobile phase composition

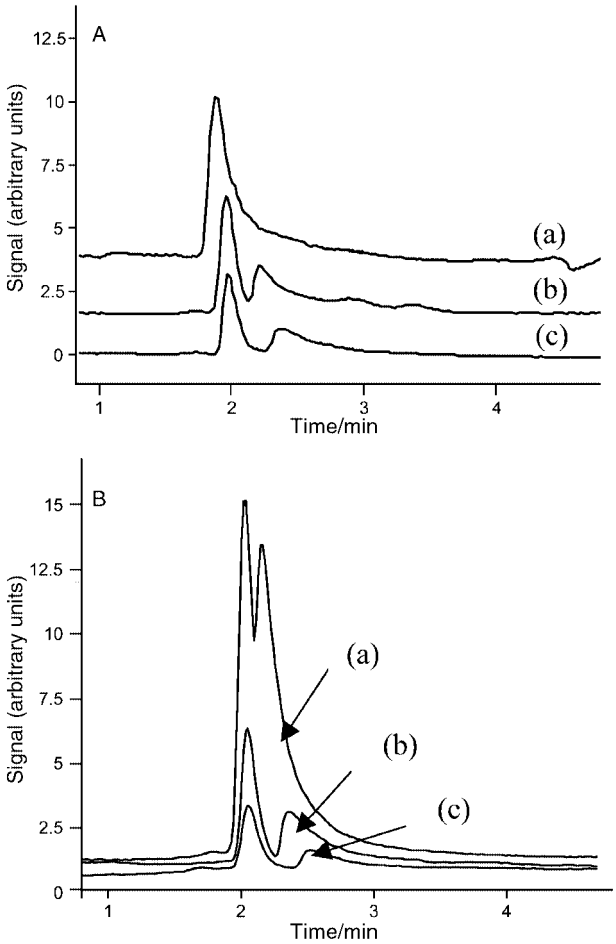

Fig. 2 (A) Influence of layer thickness on the enantiorecognition. Capillary packed with particles containing different carbon content. Assuming a homogeneous coverage of the grafted MIP layer, the carbon content corresponds to polymer thicknesses of: (a) 0.9 nm (1.6%), (b) 2.2 nm (4%) and (c) 3.8 nm (7%). Analyte: 100 µM PA racemate, mobile phase: CH_3CN : sodium phosphate buffer 10 mM pH 6.5 (70 : 30). Other conditions are given in the text. (B) Influence of analyte concentration on the enantiorecognition. Conditions: capillary packed with particles containing 7% of carbon on the surface. Analyte: PA racemate at different concentrations: (a) 500 µM, (b) 200 µM, (c) 100 µM; mobile phase: CH_3CN : sodium phosphate buffer 10 mM pH 6.5 (70 : 30). Other conditions are given in the text.

Table 3 Reproducibility of retention times and enantioselectivity. Mobile phase: CH_3CN : sodium phosphate buffer of pH 6.5 (70 : 30). Capillaries packed with particles containing 7% of carbon on the surface. PA racemate concentration: 200 µM

	t_{R1}/min		t_{R2}/min		α	
	Mean value	RSD (%)	Mean value	RSD (%)	Mean value	RSD (%)
Intraday n = 9	1.193	0.89	2.176	1.06	1.542	0.84
Interday n = 9[a]	1.909	2.63	2.137	3.56	1.545	0.91
Intercapillary n = 9[b]	2.174	7.75	2.435	7.13	1.537	0.95

[a] Three replicates on three consecutive days. [b] Three capillaries (three replicates each).

and of silica porosity on both the EOF formation and on the enantioselectivity. The grafting technique is further excellently suited for *in situ* preparation of MIP coated capillaries or packing materials.

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