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Comparison of polymer coatings of capillaries for capillary electrophoresis with respect to their applicability to molecular imprinting and electrochromatography

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Abstract

In molecular imprinting (MI), interactive monomers and suitable cross-linking agents are polymerized in the presence of a template. Once the template has been removed, the remaining space acts as a highly specific binding site for the template or analogs thereof, due to the unique three-dimensional arrangement of interaction points. Several steps are involved in producing such a polymer coat inside a capillary electrophoresis capillary. First, the silanization of the inner surface of the capillary with a suitable silane is necessary, to link a monolayer of unsaturated groups suitable for polymerization to the capillary surface. These monomeric groups are then integrated into the three-dimensional polymer coat produced in the next step. MI-capillary coatings ideally are highly porous and of a thickness, δ, which is smaller than the inner radius, r, of the capillary in question. Porous polymer networks can be produced by dispersive polymerization using a suitable solvent (porogen). However, the exact conditions for producing a coating suitable for capillary zone electrophoresis had to be determined experimentally. Seven porogens, namely hexane, toluene, tetrahydrofuran, acetonitrile, $CHCl_3$, dimethyl sulfoxide and dimethylformamide, and two cross-linkers, namely ethyleneglycoldimethacrylate and divinylbenzene, at concentrations of between 5 and 20% (v/v) were investigated. In about 20% of the combinations, a polymer coat of the desired qualities was obtained. The applicability of the MI capillaries to specific separations was demonstrated for the separation of a racemic mixture of *S*(+)- and *R*(−)-2-phenylpropionic acid. *trans*-3-(3-Pyridyl)-acrylic acid was used as the interactive monomer in this case.

Keywords: Coating; Polymer coatings; Capillary columns; Molecular imprinting; Phenylpropionic acid

1. Introduction

It is common to see the high specificity of biological interactions symbolized by the lock and key analogy. The physical basis for the finely tuned interactions observed is concomitantly seen in the necessity for a 'perfect' fit of a number of sterically arranged interaction points at the respective binding sites. In the past, it has been shown that this type of specific molecular recognition is not a prerogative of nature but can be artificially produced by 'molecular imprinting' (MI) and 'molecular footprinting' (MF), respectively [1–5].

Molecular imprinting, see Fig. 1, is a technique whereby three-dimensional, cross-linked polymer networks bearing distinct interaction points are created in the presence of a template. The interaction points are provided by a so-called interactive monomer, which is incorporated into the network as determined by the template. Thereby, the template

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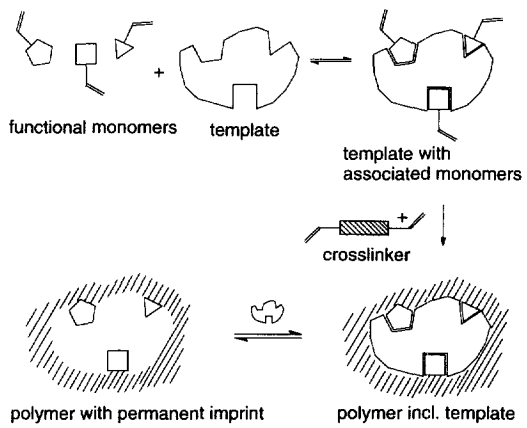

Fig. 1. The principle of molecular imprinting.

('analyte') creates a specifically 'imprinted' groove in the polymer. After the template has been removed, the remaining molecularly imprinted polymer (MIP) possesses a permanent memory of the original template in terms of complementary shape and chemical functionality. Just as its biological counterpart, the artificial interaction site specifically 'recognizes' the target molecules, or an analog thereof, even in a complex solution, and/or is able to differentiate between the template and its optical or structural isomer.

Two approaches to the formation of the complex between the template and the functionalized monomer are possible. In non-covalent imprinting, associations based on electrostatic, hydrophobic, dipole–dipole interactions, chelating groups, hydrogen bridges, etc., are used [6–8]. The flexibility of the template–monomer complex during the imprinting reaction is comparatively high in this approach, although a non-uniform distribution of binding site affinity is the inevitable consequence. In covalent imprinting, as suggested by Wulff [9] and Wulff and Schauhoff [10], labile covalent bonds, such as boronate esters, Schiff's bases, etc., are formed between the template and the monomer. Covalent imprinting has been highly successful in the case of molecules bearing 1,2- and 1,3-diol groups, e.g. various sugar derivatives. The method is limited by the binding chemistry, however, since only a small number of reactions and functionalized groups are known to give the required reversible covalent bonds.

The majority of MIP are currently designed for the chromatographic separation of racemic mixtures. However, chemically speaking, MIP are robust polymers and such mimics of biospecific interaction sites should therefore be quite stable under in vitro analysis and regeneration conditions, which are unsuitable for biological affinity mediators. An area where MIP could thus become extremely useful is that of capillary electrophoresis (CE), where currently only the unspecific separation of analytes due to velocity differences in the electrical field is possible. The combination of MI and CE would extend the range of this powerful analytical technique, by a method based on the pre-determined recognition of the analyte (affinity CE, ACE).

Presently, ACE is still in its infancy. The majority of the pertinent publications describe homogeneous, non-competitive assays, where expensive immunoreagents have to be added in excess to each individual sample [11–13]. The setting up of a heterogeneous ACE would require the immobilization of the affinity mediator within the capillaries, e.g. in a cross-linked gel matrix. Enantiomer separation, using a selector (β-cyclodextrin) immobilized in a cross-linked gel, has been described previously by Cruzado and Vigh [14]. As far as one can judge from the experience gained with cross-linked gels in general and from attempts to immobilize biologically active proteins in such gels in particular, a 'conventional' ACE is doomed to fail, due to the instability of the affinity gels under CE conditions (temperature gradients, bubble formation, high voltages of up to 30 kV, extreme buffer pH, the impossibility of using pressure for sample injection or to exchange the gel, etc.). The low migration velocity of larger molecules in cross-linked gels is another foreseeable problem, since in CE, contrary to conventional slab gel electrophoresis, all molecules have to pass the detection window.

Previous attempts to combine the concepts of MI and CE resulted in capillaries filled in toto with the MIP [15–17]. However, as explained above, such polymer-filled capillaries have distinct disadvantages. The goal of the proposed research was to investigate the possibility of producing an inner wall coat of defined thickness within the capillary. Thereby, a robust capillary zone electrophoresis (CZE) technique is established, which is capable of utilizing specific molecular recognition of the analyte while being independent of the problems typical for both biological affinity mediators and capillary gel elec-

trophoresis. The fact that such coatings can indeed support specific interactions is demonstrated by a separation of a racemic mixture of *S*(+)- and *R*(−)-2-phenylpropionic acid.

2. Experimental

2.1. Chemicals

Fine chemicals were from Sigma and bulk chemicals were from Fluka. The highest available quality was used throughout.

2.2. Templates

S-(+)-2-Phenylpropionic acid and *R*-(−)-2-phenylpropionic acid were dissolved in a mixture of methanol and high purity water (1:1, v/v). The racemic mixture (1:1, v/v) was prepared in the same solvent. The concentration of the respective enantiomers was 0.05% (v/v) in all samples.

2.3. Capillary activation

Prior to the actual coating, the capillaries were rinsed with 1 *M* NaOH for 5 min and kept at room

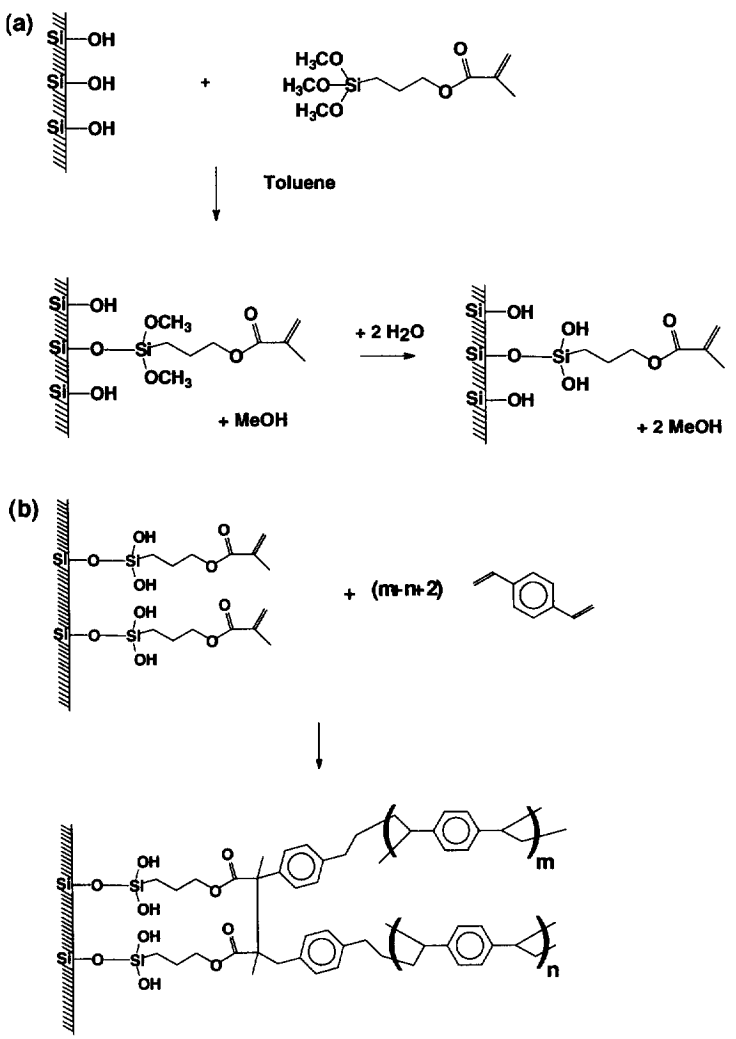

Fig. 2. Steps involved in the coating of the inner silica surface of a CE capillary.

temperature, filled with the alkaline solution, for another 10 min. Afterwards, the capillaries were treated with consecutive rinses of water (2 min), 1 *M* HCl (2 min), water (5 min) and, finally, they were dried in an air stream (5 min). Then the capillaries were rinsed for 2 min with pure toluene. Afterwards, a 10% solution of the silane (3-methacryloxypropyltrimethoxysilane) in toluene was pumped through the capillaries for 10 min. The silane solution was left within the capillary for another 2 h at room temperature. The capillaries were again rinsed for 5 min with toluene to remove the excess silane and finally were dried in an air stream for 1 min.

2.4. Capillary coating

For the polymerization, silane-activated capillaries were filled with the reaction mixture. This mixture contained the monomer(s) and 1 mol.% of the initiator of the radical polymerization (azo-*bis*-cyclohexanecarbonitrile) in their respective solvents. After rinsing the capillary with this solution (5 min), it was placed, encased in a protective glass tubing, in an oven (65°C). The polymerization reaction was allowed to proceed for 48 h. To prevent the solvent from evaporating, the ends of the capillaries were sealed with glue (Araldite Rapid, Ciba) after the reaction mixture had been added. These ends were cut off afterwards. The remainder of the reaction mixture was flushed out and the capillary was rinsed with toluene for 5 min, dried in an air stream for 2 min and finally rinsed with water (5 min) prior to storage. To prevent the polymer coat from drying out, the capillaries were kept wetted with water between experiments.

Early experiments were carried out with either of the two investigated cross-linkers, EGDM (ethyleneglycoldimethacrylate) and DVB (divinylbenzene), as sole provider of the polymerizable group. In later experiments, a mixture of the interactive monomer and the cross-linker was used at a certain ratio. The majority, i.e. 85% by mole, of the monomeric units in the reaction mixture were provided by the cross-linker, the remaining 15% were provided by the interactive monomer. In the imprinting experiments, 5 mol.% of the template were added.

2.5. Electron microscopy

Scanning electron microscopy (SEM) pictures were taken of some short (2 mm) pieces cut arbitrarily from the capillaries. An Hitachi S-570 scanning electron microscope was used.

2.6. Capillary electrophoresis

CE analysis was performed on a Hewlett-Packard 3D-CE instrument. For data collection, data analysis and for system control, the HP 3D-CE (Rev. A01.02) was applied. Detection was by UV absorbance with a photodiode array detector (range 190–350 nm). Capillaries were from CS-Chromatographie Service (Langerwehe, Germany) and from Bio-Rad (Munich, Germany).

The following conditions were used unless mentioned otherwise. Capillaries had an effective length (inlet to detector) of 35 cm, corresponding to a total length of 43.5 cm. Inner diameters were 100 μm

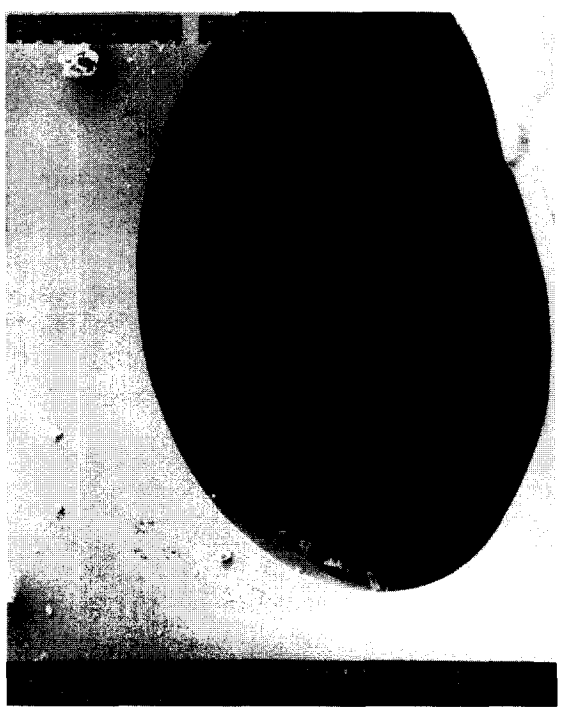

Fig. 3. EM of a silica capillary treated with 3-methacryloxypropyltrimethoxysilane (10%, v/v). The capillary diameter was 100 μm and the solvent was toluene. The coat thickness is below 0.1 μm.

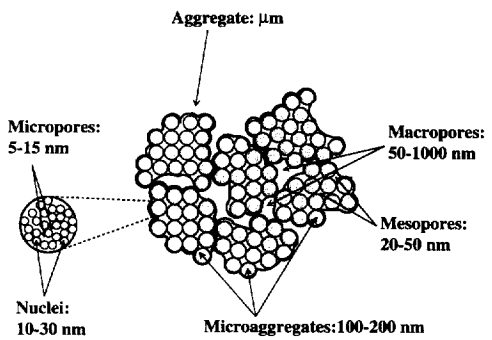

Fig. 4. Principle of forming highly porous three-dimensional polymer networks through dispersive polymerization.

throughout. A temperature of 25°C and a voltage of +10 kV (67 µA) were maintained. The injection was by pressure, i.e. 50 mbar for 3 s. The electrophoresis buffer was 50 m*M* NaH_2PO_4 (pH 4.65). The detection wavelength was 200 nm. Prior to each run, the capillaries were pre-conditioned by rinsing them with 100% methanol (1 min), followed by methanol–water (1:1, v/v) (1 min), water (1 min) and, finally, electrophoresis buffer (2 min). Equilibration of the capillary was done by filling it with buffer for 5 min and waiting for 20 min.

3. Results and discussion

The combination of MI and CE is of specific appeal, since it is already common to coat the inner capillary walls with, much thinner, polymer films, e.g. to prevent protein adsorption or suppress the electroendosmotic flow. However, in spite of the acknowledged importance, the best methods for producing a stable and smooth polymer film within the capillaries is still under discussion.

The exact procedure for imprinting capillaries involves several steps, each of which has to be optimized individually (see Fig. 2).

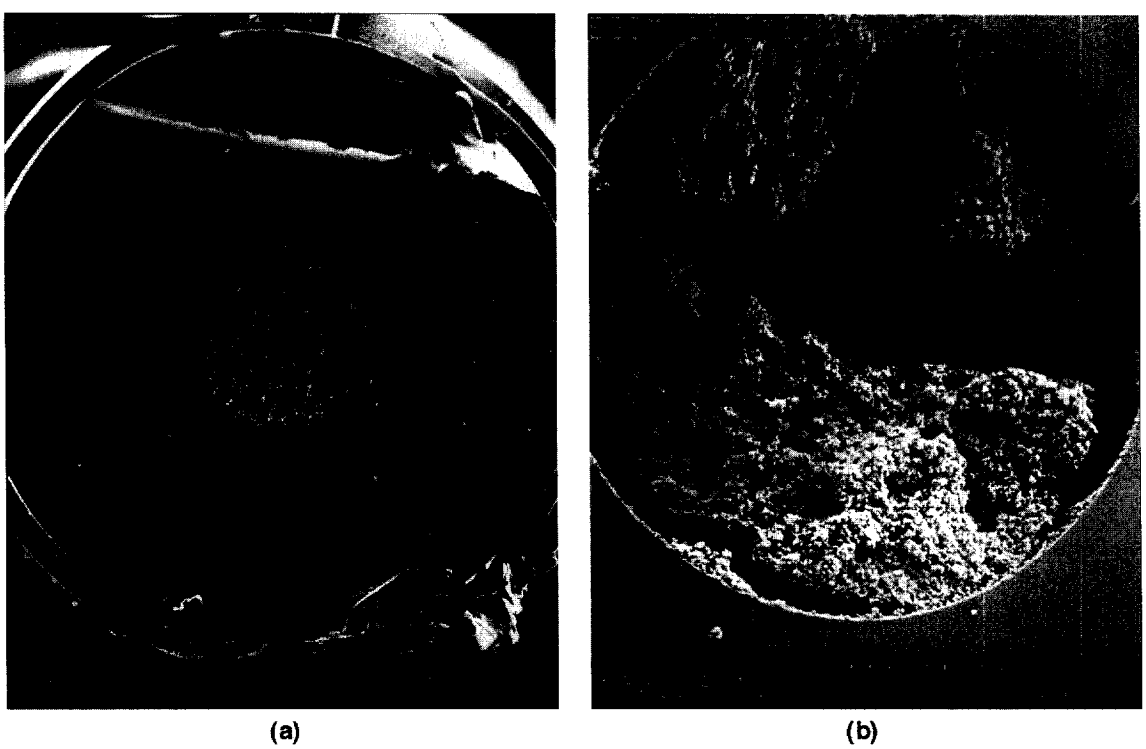

Fig. 5. EM of CE capillaries 'coated' with EGDM. (a) 15% (v/v) EGDM in toluene and (b) 20% (v/v) EGDM in dimethyl sulfoxide (DMSO). The diameter of the capillary was 100 µm in both cases.

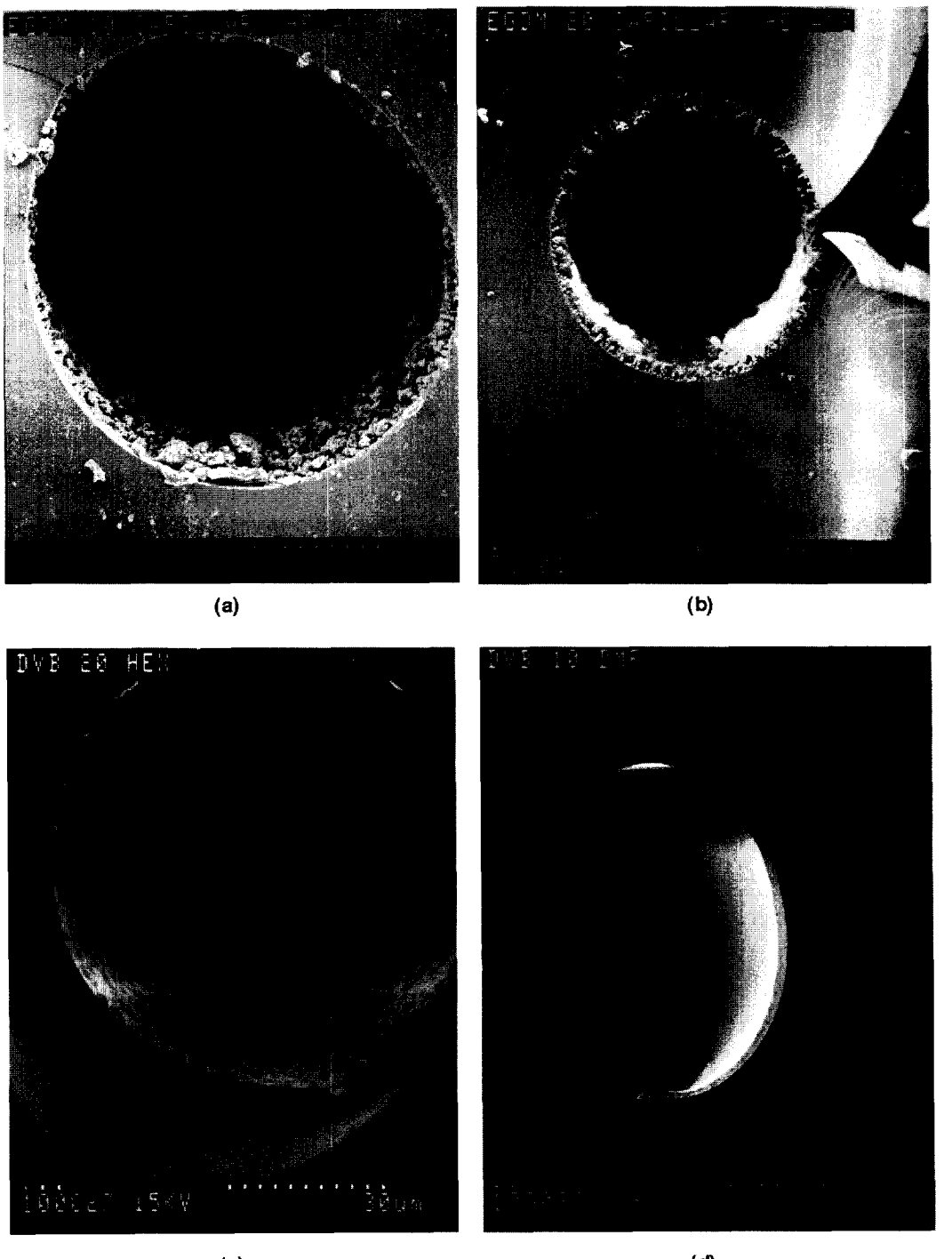

Fig. 6. EM of successfully coated CE capillaries. (a) 10% (v/v) EGDM in hexane; thickness of the coat, 2 μm; (b) 20% (v/v) EGDM in hexane; thickness of the coat, 6 μm; (c) 20% (v/v) DVB in hexane; thickness of the coat, 2.7 μm; (d) 10% (v/v) DVB in dimethylformamide (DMF); thickness of the coat, 3 μm. The diameter of the capillary was 100 μm in all cases.

3.1. Pre-activation of the capillary surface by an unsaturated silane

The first step entails the activation of the inner capillary surface (silanol groups) with an unsaturated silane, thereby introducing polymerizable groups for the later coating. Chemically speaking, this part of the process is rather straightforward, however, it is decisive later for the stability of the linkage of the MIP to the capillary wall. In this context, we find similarities to the chemistry proposed for the generation of capillary coatings in general and that suggested, e.g., as a first step for the imprinting of silica particles for chromatographic purposes. In particular, the silica surface is covered with a monolayer of the silane. Since only a monomolecular layer will be produced, reactions suggested in the literature for polymer coatings can be used [18]. In our case, the 3-methacryloxypropyltrimethoxysilane commonly used for the purpose gave good results in the preliminary experiments. As expected, the activated capillaries do not look different from the 'naked' silica ones under the electron microscope (Fig. 3).

3.2. Synthesis of polymer coating of controllable thickness within the capillaries

The next step theoretically involves the actual imprinting of the capillaries and here, things begin to differ from both the 'standard' coating of CE capillaries and the 'standard' imprinting, e.g., of silica-based high-performance liquid chromatography (HPLC) phases. While we expected the actual imprinting process to be not too different from that employed e.g. in HPLC, rather strict boundary conditions had to be observed in the case of the polymer coating produced within the capillaries. This coat had to have a 'three-dimensional' quality, in order to allow the stereoselective interaction. It should also be highly porous, to facilitate the attach-

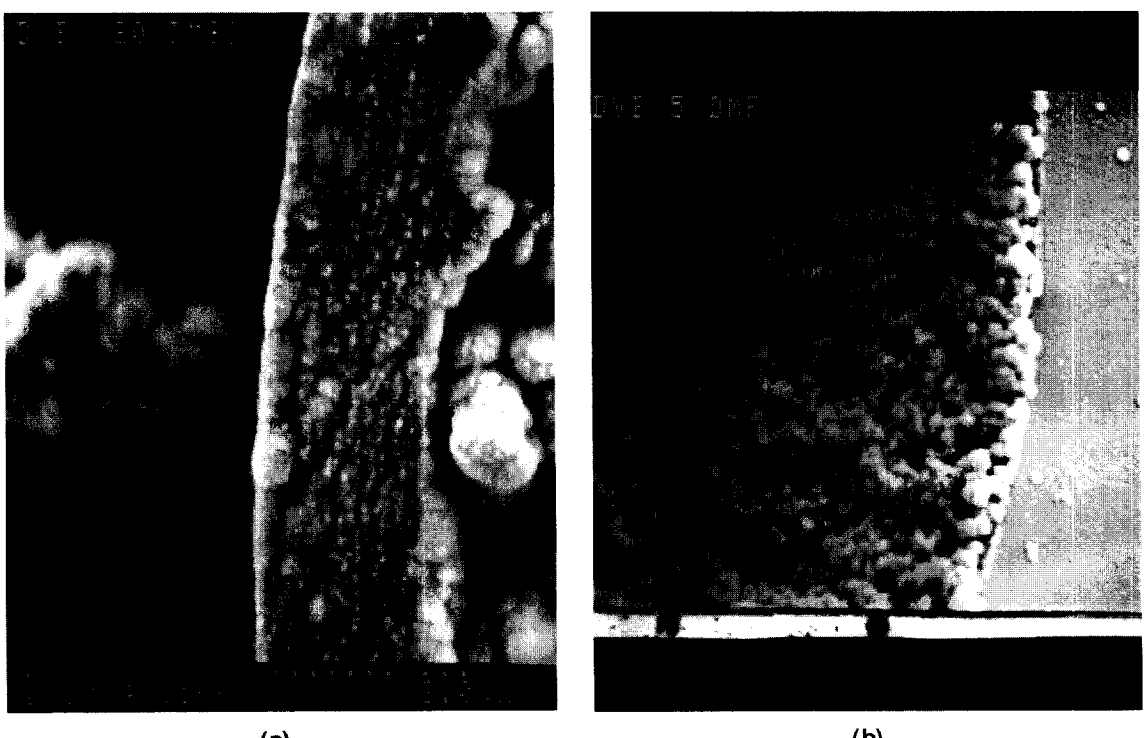

Fig. 7. EM of successfully coated CE capillaries: (a) 20% (v/v) DVB in DMSO; thickness of the coat, 3 μm; (b) 5% (v/v) DVB in DMF; thickness of the coat, 0.8 μm. The diameter of the capillary was 100 μm in both cases.

ment and detachment of the analyte molecules. On the other hand, we did not want to create a capillary filled in toto with the imprinted polymer. Our past experience has taught us that when capillaries are filled with a stably attached cross-linked gel, this can cause extreme difficulties in the actual CE application (bubble formation interrupting the electric field, washing and sample injection by pressure not being possible, movement of large analytes being extremely slow, etc.) [19]. In addition, the imprinted capillaries still needed to be compatible with the detector, i.e. the UV absorbance of the polymer coating must not be too high.

The protocols for producing such a thick, but not too thick, polymer coat on top of a silica surface had to be developed empirically. A method to create highly porous polymer networks is dispersive polymerization. In this case, the monomer molecules are dissolved in a suitable solvent at a concentration that is not too high. The applied solvent is not capable of dissolving the corresponding polymers as well. As a result, the polymer's coils collapse and precipitate out as they form. The collapsed structures will then assemble into larger and larger highly porous aggregates (Fig. 4). The density and structure of the final polymer is highly dependent on the chemical nature of the solvent (porogen).

Once the basic chemistry has been decided upon, a suitable cross-linker has to be chosen. A cross-linker needs to carry at least two polymerizable groups in order to enable the formation of a three-dimensional structure. The length and the rigidity of the cross-linker have important consequences for the structure and rigidity of the resulting polymer network. In our case, two cross-linkers and several solvents were studied. The influence of the cross-linker concentration in combination with the different solvents on the quality of the coatings (smoothness, homogeneity, stability) was systematically investigated.

As shown in Fig. 5, standard polymer synthesis conditions may easily lead to blocked capillaries, while others yield the desired coatings (see Fig. 6).

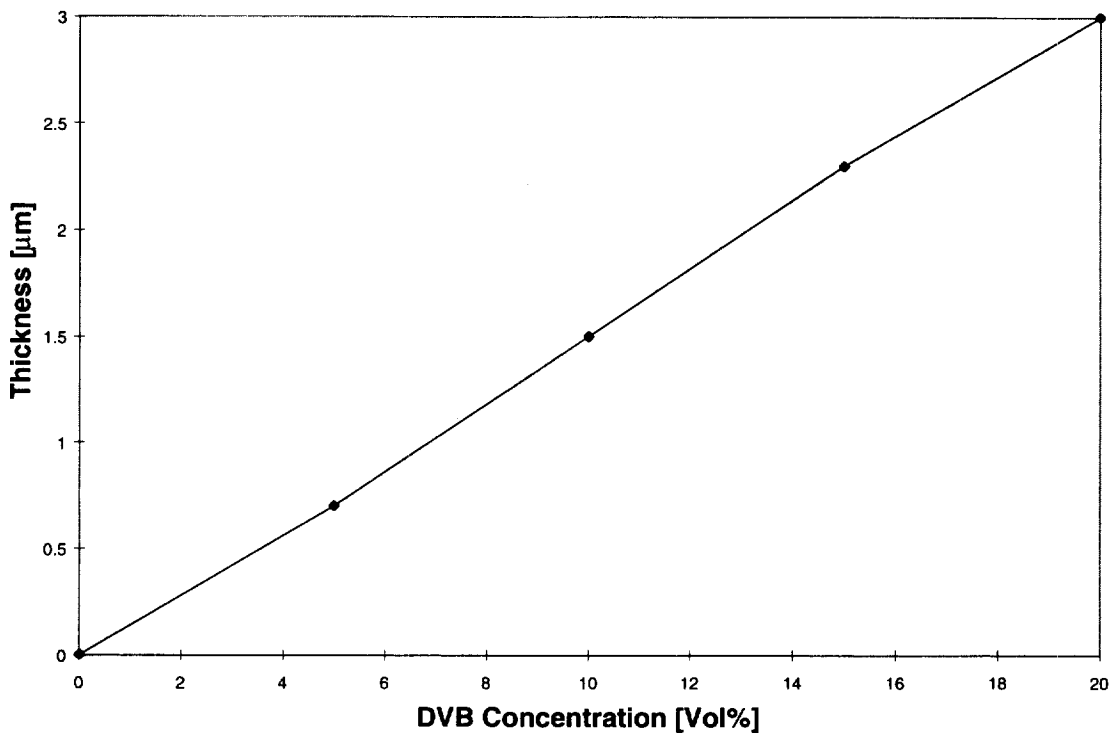

Fig. 8. Coat-thickness as a function of the concentration of the cross-linker. The cross-linker was DVB and the porogen in this case was DMSO. The diameter of the capillary was 100 µm in all cases.

Table 1
Summary of the experimental results, hatched areas indicate conditions that resulted in the formation of satisfactory coatings, black areas mark conditions that were not included in the investigations

Monomer		Solvent / Porogen						
Type	Content	Hexane	Toluene	THF	ACN	$CHCl_3$	DMSO	DMF
EGDM	5 Vol%							
	10 Vol%							
	15 Vol%							
	20 Vol%							
DVB	5 Vol%							
	10 Vol%							
	15 Vol%							
	20 Vol%							
DVB / VP	5 Vol%							
	10 Vol%							
	15 Vol%							
	20 Vol%							

The monomer–solvent combinations used are detailed below each picture. Even in the successful experiments, differences in the macroscopic structure can be observed, e.g. smooth layers and cases where a fairly dense layer is topped with a much looser amorphous and highly irregular layer. The detachment of the polymer coat from the capillary, on the other hand, which can be seen in some cases, is an artifact produced by the EM conditions (high temperatures required during the gold coating procedure prior to the EM measurements). Such a detachment is extremely unlikely during the normal CE application of the capillaries.

As shown in Fig. 7, the polymer coat is highly porous, as desired. The thickness of the coating

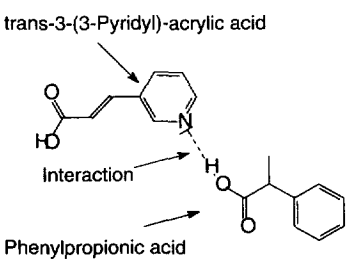

Fig. 9. Interaction between *trans*-3-(3-pyridyl)-acrylic acid and 2-phenylpropionic acid.

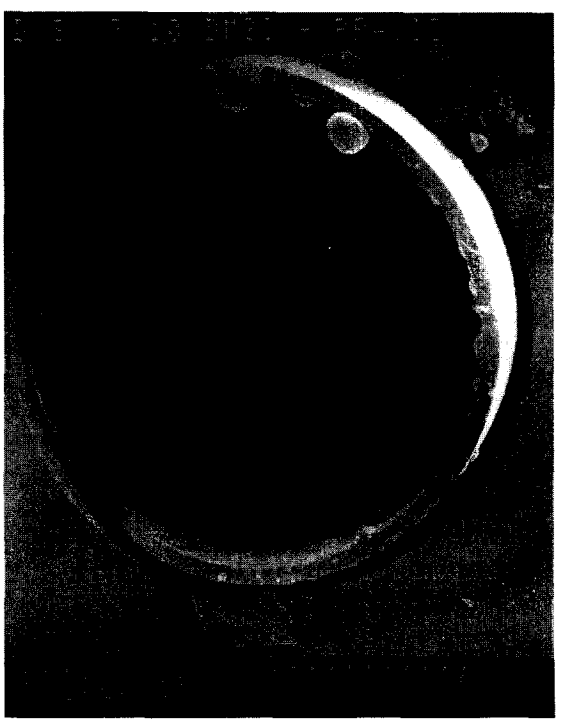

Fig. 10. EM of an imprinted CE capillary 10% (v/v) polymerizable groups in DMSO; ratio of the cross-linker to interactive monomer, 85:15; template, *S*(+)-2-phenylpropionic acid 5 mol.%; thickness of the coat, 4 μm; capillary diameter, 100 μm.

produced in a given experiment can be influenced by the cross-linker concentration, since we observed a linear relation between the two (Fig. 8).

The results of the experiments are summarized in Table 1. Cells marked by hatching indicate conditions that gave excellent results in terms of a stable, uniform capillary coating.

3.3. Synthesis of a polymer film that is capable of molecular recognition

For each template, a suitable interactive monomer needs to be chosen. As model templates for these studies, the easily detected enantiomers *S*(+) and *R*(−)-2-phenylpropionic acid were selected. The substance is stable and predictions concerning the interactive monomer were possible. *trans*-3-(3-Pyridyl)-acrylic acid can, for example, fulfil the purpose in this case (Fig. 9).

The ratio of the interactive monomer to the total number of monomeric units in the polymer coat is an important parameter in MI, which, at present, also has to be determined empirically for each individual template. A molar ratio of 85:15 of the polymerizable groups provided by the cross-linker and the interactive monomer was found to be optimal in our case, since it provides a sufficient number of interaction points while yielding coatings that cannot be

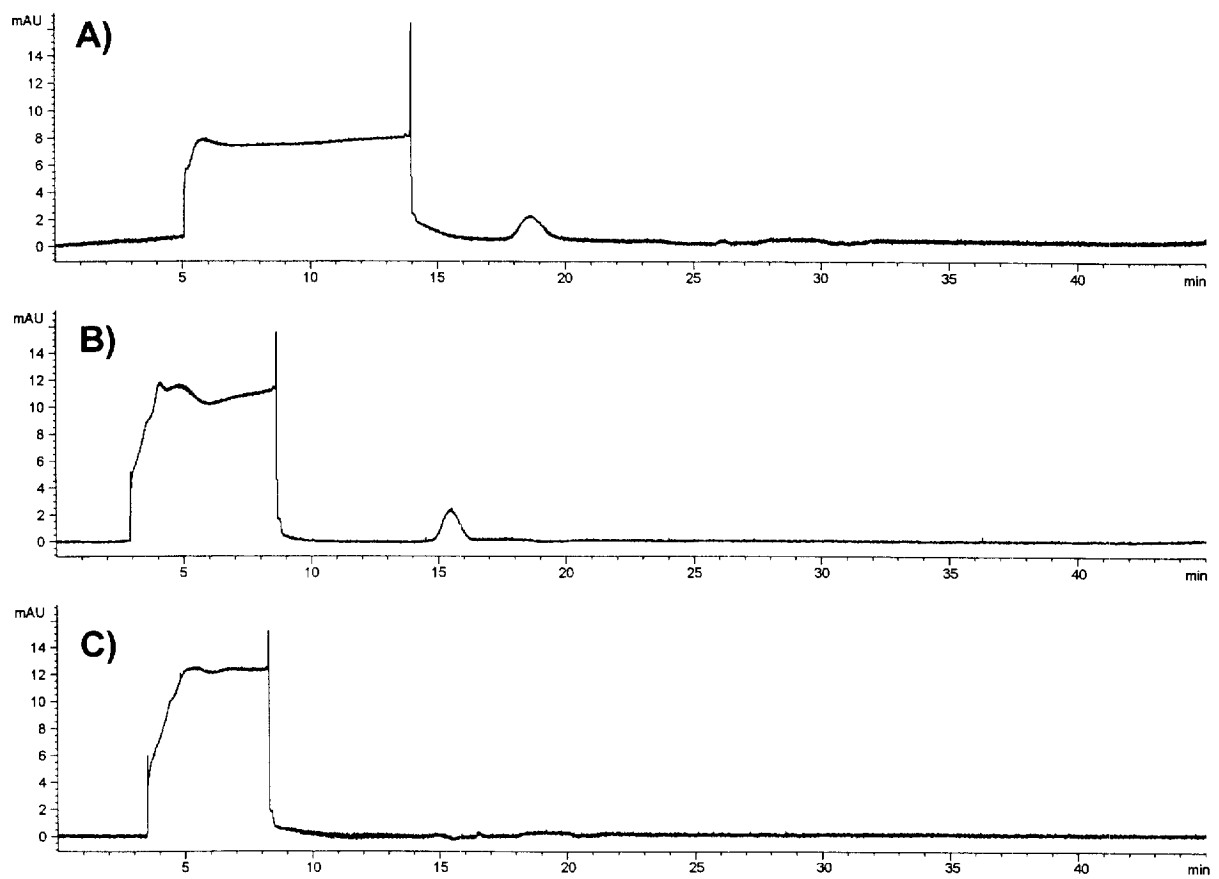

Fig. 11. Electropherograms of 2-phenylpropionic acid using capillaries imprinted with *S*(+)-2-phenylpropionic acid. (A) Racemic mixture; (B) *R*(−)-2-phenylpropionic acid; (C) *S*(+)-2-phenylpropionic acid. Coating parameters: Porogen, DMSO; cross-linker, DVB; interactive monomer, *trans*-3-(3-pyridyl)-acrylic acid; 5% (v/v) polymerizable groups; ratio cross-linker–interactive monomer, 85:15; template, *S*(−)-2-phenylpropionic acid (5 mol.%); thickness of the coat, 0.8 μm. CE conditions: capillary length, 43.5 cm (inlet to detector, 35 cm); inner diameter, 100 μm; coat thickness, 3 μm; temperature, 25°C; buffer, 50 m*M* NaH_2PO_4 (pH 4.65); detection wavelength, 200 nm; applied voltage, 10 kV.

differentiated in terms of the pressure drop of the EM pictures from the coatings produced with only the cross-linker present (Fig. 10). The template, *S*(+)-2-phenylpropionic acid, was added at a molar ratio of 5% in this case.

3.4. Separation of a racemic mixture

The imprinted capillaries were used to separate a racemic mixture of *S*(+)- and *R*(−)-2-phenylpropionic acid. In the electropherogram (Fig. 11A), a single peak is seen. When the two components of the racemic mixture are injected separately, only the *R*(−)-2-phenylpropionic acid is detected (Fig. 11B). Due to the strong interaction, the template, *S*(+)-2-phenylpropionic acid, elutes in a very broad peak, which can barely be distinguished from the baseline noise (Fig. 11C). A close inspection of Fig. 11A reveals that a broad peak, presumably caused by the template, can also be seen here.

The CE conditions obviously need to be improved further. The strong retention may be reduced by, for example, lowering the pH value of the buffer. However, the possibility of producing an imprinted coating that is several μm thick, rather than a polymer-blocked capillary, and thereby of retaining both the advantage of CZE and the high and specific affinity of ACE has been demonstrated in principle.

4. Conclusion

It can be deduced from these preliminary experiments that it is indeed possible to produce a three-dimensional imprinted polymer coat of controllable thickness on the inner capillary wall rather than blocking the capillary totally, even when a bifunctional monomer is used in excess. The production of a capillary of predetermined selectivity by purely chemical means is, therefore, possible. However, at all stages, basic problems and questions remain, which must be investigated and resolved before this new approach to ACE can become a viable option in analytical chemistry.

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