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Development of reactive thiol-modified monolithic capillaries and in-column surface functionalization by radical addition of a chromatographic ligand for capillary electrochromatography

Beatrix Preinerstorfer, Wolfgang Bicker, Wolfgang Lindner, Michael Lämmerhofer*

Christian Doppler Laboratory for Molecular Recognition Materials, Institute of Analytical Chemistry, University of Vienna, Währingerstrasse 38, A-1090 Vienna, Austria

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Abstract

Reactive thiol-modified capillary columns for capillary electrochromatography (CEC) were developed by transforming the pendent 2,3-epoxypropyl groups of poly(glycidyl methacrylate–*co*-ethylene dimethacrylate) (poly(GMA–*co*-EDMA)) monoliths into 3-mercapto-2-hydroxy-propyl residues by a nucleophilic substitution reaction, employing sodium-hydrogen sulfide as nucleophilic reagent. Conditions for this modification reaction were systematically optimized with respect to different parameters, such as reaction temperature, pH-value, reaction time, type and concentration of organic modifier, and concentration of the sodium-hydrogen sulfide solution. The amount of thiol groups that was generated on the monolith surface was determined directly in the capillaries by a disulfide-exchange reaction employing 2,2′-dipyridyl disulfide (DPDS). This reaction in the capillary liberates pyridine-2-thione in equimolar amount to the surface sulfhydryls, which was collected into a vial and determined photometrically at 343 nm by RP-HPLC. About 17% of the total lateral epoxide moieties of the monolithic substrate could be transformed to reactive sulfhydryl groups, which corresponds to about 0.7 mmol g^{-1} monolithic polymer, with a column-to-column repeatability of 3.2% R.S.D. The reactive thiol groups can be utilized to attach any chromatographic ligand with appropriate anchor in a second step, e.g. by radical addition, graft polymerization, nucleophilic substitution, disulfide formation or Michael addition reaction. To demonstrate the feasibility of the concept, we chose an anion exchange type chromatographic ligand based on a quinine derivative, *O*-9-*tert*-butylcarbamoylquinine (*t*-BuCQN) which was attached to the monolith in a radical addition reaction, for a further in-column surface functionalisation. About 78% of the sulfhydryl groups were derivatized with *t*-BuCQN as determined from differential DPDS assays before and after the selector immobilization reaction. The applicability of these surface-functionalised monolithic capillary columns could be shown by an electrochromatographic separation of the enantiomers of *N*-3,5-dinitrobenzoyl-leucine, which performed fairly well compared to an analogous capillary that was fabricated by an in situ copolymerization approach.

Keywords: Monolithic columns; Stationary phases, electrochromatography; Enantiomer separation; Electrochromatography

1. Introduction

Capillary electrochromatography (CEC) combines the high peak efficiency of capillary zone electrophoresis (CZE) with the extraordinary versatility regarding tailoring of selectivity which is typical for high-performance liquid chromatography (HPLC) [1]. Despite this major advantage CEC has not yet gained as much importance as separation technique like HPLC or CZE. Along this line, a serious problem is the fabrication of dedicated capillary columns with tailored surface chemistries for the varieties of chromatographic modes and suitable electroosmotic flow. At present most columns employed in CEC are still fused-silica capillaries packed with functionalised silica particles. Performance and stability of such particulate packings strongly depend on the integrity of the packing and, in particular, of the retaining frits at both ends of the packed bed. The packing procedure is tedious and the fabrication of the frits is often not very well reproducible. For this reason, monolithic columns are of growing interest in CEC [2,3], because of their straightforward fabrication. Also the

* Corresponding author. Tel.: +43 1 4277 52323; fax: +43 1 4277 9523.
E-mail address: michael.laemmerhofer@univie.ac.at (M. Lämmerhofer).

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column-to-column reproducibility is quite acceptable and through copolymerization with active groups on the internal capillary surface, which leads to crosslinking of the monolith body to the capillary wall, the necessity of retaining frits to support the bed is eliminated. Other advantages include the optimal compatibility with mass spectrometric detection and the appealing chromatographic performance for (bio)macromolecules. Today for (electro)chromatography most interest is focused on the area of porous siliceous [4], styrenic [5–7] and acrylic monoliths [8–12].

From a chemical viewpoint, the typical monolith for (electro)chromatography consists of a crosslinked polymer backbone that supports the morphological structure and carries pendent functional groups which represent the interactive moieties providing the dedicated selectivity. For CEC, ionizable residues are in addition required for electroosmotic flow (EOF) generation. There are two fundamentally different ways for the manufacturing of such functionalised monolithic capillary columns: (i) one possibility is the in situ preparation of functionalized monoliths in a fused-silica capillary by co-polymerization of a functional monomer with the interactive residue. This is the preferred methodology for the preparation of organic polymer monoliths for reversed-phase [9] and ion-exchange chromatography [13], but also for more advanced chromatographic modes such as enantioselective [14–19] and even affinity-type chromatography [20]. The chromatographic performance and hence the success of this approach strongly depends on a proper choice of porogenic solvents in the polymerization mixture. A considerable disadvantage of this process is that even minute changes in the composition of the polymerization mixture may affect the resulting pore structure. Attempts to incorporate different chromatographic moieties by using a single recipe with identical co-monomer, crosslinker, and porogen composition, but replacing solely the functional monomer yet leading to optimal morphology ultimately fail. Thus, every new chromatographic entity to be introduced in the monolithic matrix requires a careful optimization of the pore structure [14,21].

(ii) The other option for the preparation of monolithic capillary columns is a post-modification approach, in which a (reactive) monolith is modified by in-column surface functionalisation. Several beneficial features are proprietary to this modus operandi: (1) the porous structure of a reactive monolith that allows a subsequent chemical modification has to be optimized only once and can be used as substrate for the immobilization of various dedicated chromatographic ligands. (2) After adequate surface activation any ligand with suitable anchor can be grafted on the monolith and the properties of these capillaries can readily be compared since the porous structure and morphology are the same or similar. (3) All the bonded ligands are exposed to the interface between stationary and mobile phase, and therefore fully available for chromatographic interactions. In contrast, it has to be kept in mind that a significant percentage of chromatographic moieties is buried inside the polymer globules in case of in situ prepared functionalized monoliths. (4) Because of the very low total volume of the capillary columns (e.g. 100 μm i.d.) microlitre volumes of reaction solutions and hence only small quantities of ligand are needed, much less than with the in situ approach. This opens the avenue for the use of precious ligands such as chiral selectors or affinity ligands.

Post-synthesis functionalization procedures are in particular the methods of choice for silica monoliths prepared by sol–gel technology [4,22–24], but have also been reported for organic polymer monoliths in capillary, μ-chip, disk, or standard column format. Two complementary strategies are in principle amenable for the introduction of chromatographic ligands by the post-modification of organic polymer monoliths which is the topic of the present study: In one case, a single chromatographic moiety per reactive group on the monolith surface is attached by dedicated chemistry leading to a well-defined or well-controlled chemical modification (brush-type phases). Using this concept both low-molecular [6,25–28] and macromolecular ligands [29–32] have been covalently bonded to reactive monoliths, e.g. via epoxide [25–27,29,31,32], azlactone [30], or chloromethyl groups [6] as reactive anchor on the monolith by their reaction with amino groups of the chromatographic ligand.

On the other hand, by grafting of polymer chains onto the monolith surface multiple functionalities per reactive group or site may be introduced leading to tentacle-like phases with higher capacity. Reported examples of the latter approach include the grafting of poly(2-acrylamido-2-methyl-1-propane sulfonic acid) chains onto the surface of a hydrolyzed poly(glycidyl methacrylate–*co*-ethylene dimethacrylate) (poly(GMA–*co*-EDMA)) monolith using cerium(IV) initiated free-radical polymerization [33], the graft polymerization of *N*-isopropylacrylamide onto allylamine-modified epoxy monoliths [34], the photografting of porous polymer monoliths with 2-acrylamido-2-methyl-1-propane sulfonic acid [35] and 4,4-dimethyl-2-vinylazlactone [36], the grafting of styrene and methacrylate monomers onto porous monoliths with alkoxy amino initiator prepared by living free-radical polymerization [37], and the grafting of various functional monomers onto monoliths prepared by living ring-opening metathesis polymerization [38].

The aim of our work was to modify the epoxide groups of a poly(GMA–*co*-EDMA) monolith in such a way that reactive thiol groups are generated which can be utilized to attach a chromatographic ligand in a second step by versatile chemistries, e.g. by radical addition, graft polymerization, nucleophilic substitution, disulfide formation and Michael addition reaction. It is obvious that an in situ fabrication of a reactive thiol monolith by direct copolymerization with a functional monomer having a free sulfhydryl group is not feasible, because the thiol group would interfere with the polymerization. The functional thiol-containing monomer would act like a crosslinker so that the sulfhydryl would not be available anymore after the polymerization. A post-synthesis modification strategy was therefore envisaged.

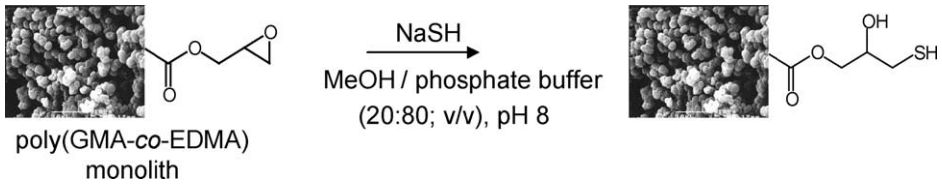

Fig. 1. Reaction scheme of surface modification for the preparation of reactive thiol monoliths.

Thus, to modify the surface of a poly(GMA–*co*-EDMA) monolith the existing epoxide groups were transformed to 3-mercapto-2-hydroxy-propyl residues in a nucleophilic substitution reaction, employing sodium-hydrogen sulfide in aqueous media as nucleophilic reagent (see Fig. 1). The surface functionalization reaction was systematically optimized by studying the effect of the following parameters: reaction temperature, pH-value, reaction time, type and concentration of organic modifier in the reaction media, and concentration of the sodium-hydrogen sulfide solution.

In the course of this approach, the precise and accurate characterization of the yield of the reaction on the solid surface arises as a major difficulty. This is in general a critical issue of all approaches where in situ prepared monolithic capillaries and μ-chips are modified by chemical transformation to introduce the desired chromatographic ligand by in-column or on-chip reaction. In fact, the information on the surface concentration of chromatographic moieties is most often missing. Sometimes it is substituted by data gained from polymeric material obtained from the remainder of the polymerization mixture and treated with the similar procedure of surface modification as the intact monolith in the capillary. The major shortcoming of the latter approximation is that one can never be sure of an identical yield of the reaction within the monolithic capillary and the external polymer material. For larger monolith formats such as disks and standard columns (e.g. 4 mm i.d.) the success of the surface modification can be determined by elemental analysis after removal from the column. Since the column is thereby destroyed, also this method is often not amenable or only when the separation/analysis task has been finished. On the other hand, the crosslinking of the monoliths to the capillary wall, which precludes their removal from the capillary tube, and the limited amount of material make this strategy more or less inapplicable for monolithic capillaries and μ-chips.

Herein, we propose a different and straightforward photometric methodology to determine the yield of the surface modification. The concentration of thiol groups that were generated on the monolith surface was determined by disulfide-exchange reaction using 2,2′-dipyridyl disulfide (DPDS) (see Fig. 2). In this reaction, thiols on the monolith surface are quantitatively transformed into mixed disulfides and equimolar amounts of pyridine-2-thiol being in equilibrium with the tautomeric form pyridine-2-thione, which can be determined specifically at 343 nm by photometric measurements [39–41]. In the present study, the concentration of pyridine-2-thione, which is proportional to the amount of thiol groups on the monolith surface, was separated from non-reacted DPDS employing HPLC and quantified by an external calibration curve, which was established using 2-mercaptoethanol as standard.

After thiol quantification the pyridyl disulfide monolith can be re-activated by removing the disulfides from the monolith surface with reducing reagents such as dithiothreitol (DTT), 2-mercaptoethanol, or tris(2-carboxyethyl) phosphine hydrochloride (TCEP) [41,42]. A considerable advantage of the suggested characterization methodology is its non-destructive and reversible nature. It enables a subsequent functionalization after the reactive thiol groups have been readily quantified and the active surface was reconstituted by the reductive removal of 2-pyridyl sulfide residues in the same monolithic column.

To show the applicability of the proposed approaches an enantioselective anion-exchange type chromatographic ligand based on a quinine derivative, *O*-*9*-*tert*-butylcarbamoylquinine (*t*-BuCQN) was used for a further functionalization

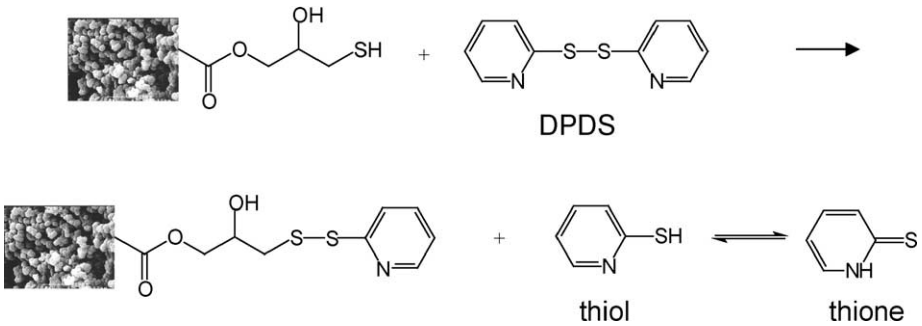

Fig. 2. Reaction scheme for the quantitative determination of thiol groups on the monolith surface using 2,2′-dipyridyl disulfide (DPDS) as chromogenic reagent.

of the monolith surface by a radical addition reaction. By employing this chromatographic ligand a direct comparison with results obtained previously [14] by the in situ incorporation procedure of the chromatographic moieties by co-polymerization with an analogous functional chiral monomer was possible.

2. Experimental

2.1. Materials and instruments

Polyimide coated fused-silica capillaries (100 μm i.d.) were purchased from Composite Metal Services (Worcester, UK). Solvents (acetonitrile, methanol) were HPLC gradient grade from Fisher Chemicals (Loughborough, UK) or Merck (Darmstadt, Germany). (*R*,*S*)-*N*-3,5-Dinitrobenzoyl–leucine (DNB–Leu) was obtained from Sigma–Aldrich. The pH was measured with a pH meter pH 540 GLP (purchased from Aigner Unilab, Vienna, Austria).

For the rinsing and washing steps in the course of the chemical modifications of the monolithic capillaries a syringe pump (Model 100, kd Scientific, New Hope, PA, USA) was utilized.

CEC experiments were carried out on an Agilent HP^{3D} capillary electrophoresis system (Agilent, Waldbronn, Germany) equipped with a diode array detector and an external pressurization system. Data processing was done with the HP^{3D} CE Chemstation software.

A Merck–Hitachi system consisting of an HPLC pump model L-7100, an autosampler model L-7200, an interface model D-7000, an UV-detector model L-7400 and a column thermostat Jetstream 2 Plus (Alltech, Unterhaching, Germany) was used for the RP-HPLC measurements.

2.2. Preparation of poly(glycidyl methacrylate–co-ethyleneglycol dimethacrylate) monolithic capillary columns

2.2.1. Capillary pre-treatment: vinylization of the inner wall of the capillary

To enhance the surface density of reactive silanol groups on the inner wall the capillary was flushed with 1 M aqueous sodium hydroxide solution (Merck, Darmstadt, Germany) for 30 min employing a Hamilton syringe and a syringe pump. After neutralization with 0.1 M hydrochloric acid (Donauchem, Vienna, Austria) (flushing for 30 min) and rinsing with water and acetone (15 min each) the silanization was carried out by flushing the capillary with a solution of 3-methacryloyloxypropyl trimethoxysilane (Höls-Petrach ABCR, Karlsruhe, Germany) in acetone (1:1 (v/v)) for 30 min. The capillary was sealed at both ends using a rubber GC septum and stored at room temperature for 12–24 h. Subsequently the capillary was rinsed with acetone for 15 min and dried by purging with nitrogen for at least 2 h.

2.2.2. In situ preparation of poly(GMA–co-EDMA) type monolithic capillaries

The polymerization mixture was prepared by merging 30% (w/w) cyclohexanol (Fluka, Buchs, Switzerland) and 30% (w/w) 1-dodecanol (Sigma–Aldrich, Steinheim, Germany) as porogenic solvents with 24% (w/w) glycidyl methacrylate (GMA; purchased from Fluka) as reactive monomer and 16% (w/w) ethyleneglycol dimethacrylate (EDMA; Sigma–Aldrich) as crosslinker. One percent (w/w) (with respect to the total monomer content) α,α′-azoisobutyronitrile (AIBN; Merck–Schuchardt, Hohenbrunn, Germany) as radical initiator was added and the mixture was sonicated (10 min at least) and purged with nitrogen (10 min). Vinylised fused-silica capillaries of a total length of about 40 cm were filled to 30 cm with the polymerization mixture employing a Hamilton syringe and sealed with rubber GC septa at both ends. The polymerization was carried out in a water bath (model M12 from Lauda, Germany) at a temperature of 60 °C for 20 h. Subsequently, the capillaries were rinsed with acetonitrile to remove the porogenic solvents and non-reacted monomers. For that purpose an HPLC pump was employed the flow of which was split with a T-connector. The split ratio was adjusted with a 50 μm i.d. open capillary on one line, while the monolithic capillary was attached on the third line.

2.3. Optimized preparation procedure for thiol-monoliths

A 2 M solution of sodium-hydrogen sulfide (Sigma–Aldrich) was freshly prepared by dissolving it in a mixture of methanol–0.1 M aqueous sodium dihydrogenphosphate (Merck), (20:80 (v/v)) and sonicating it for a few minutes. Afterwards the solution was filtered through a nylon membrane filter (Supelco, Bellefonte PA, USA). The pH was adjusted to 8.15 with diluted orthophosphoric acid (Merck).

Before surface modification the monolithic capillaries were rinsed with methanol–water (20:80 (v/v)) employing an HPLC pump. The capillaries were then flushed with 60 μl of the sodium-hydrogen sulfide solution using a Hamilton syringe and a syringe pump at a flow rate of 30 $\mu l\,h^{-1}$. After reaction (2 h) the capillaries were attached to an HPLC pump and rinsed with methanol–water (20:80 (v/v)) and then with acetonitrile.

Finally a detection window (about 3 mm) was made directly behind the monolith bed (25 cm) by removing the polyimide layer using a razor blade. The total capillary length was 33.5 cm.

2.4. Quantitative determination of the generated thiol groups on the monolith surface

The experiments for determining the thiol concentration on the monolith surface were carried out in the Agilent HP^{3D} capillary electrophoresis system. An external pressure of 8 bar at the inlet side of the capillaries was applied during the rinsing steps.

For determination of the generated thiol groups on the monolith surface the capillaries were filled with a 1.19 M solution of 2,2′-dipyridyl disulfide in acetonitrile (DPDS, "Aldrithiol-2 98%" from Sigma–Aldrich) in such a way that the entire porous polymer plug was filled with the reagent solution while the detection cell was devoid of the reagent solution. To do so first the break-through time of each capillary was determined with acetone (in acetonitrile). The disulfide-exchange reaction was allowed to occur for 1 h in the capillary. Subsequently, the capillary was rinsed with acetonitrile for 35 min and the effluent, i.e. the reaction solution was collected in a micro-vial. Two hundred microliters acetonitrile were added and the sample was transferred to an HPLC micro-vial.

Reaction product (pyridine-2-thione) and non-reacted DPDS were separated employing gradient elution RP-HPLC. The separation conditions were as follows: the stationary phase was an Agilent HP Hypersil BDS-C_{18} column, 125 mm × 4 mm i.d., with 5 μm particle size. The mobile phase consisted of water and acetonitrile and the runs were carried out by a linear gradient from 5 to 90% acetonitrile in 20 min. The flow rate was 1 ml min^{-1} and the column temperature was 25 °C. An aliquot of 20 μl of the reaction mixture was injected into the HPLC system. Reaction product (pyridine-2-thione) was detected at a wavelength of 343 nm, excessive reagent was detected at 254 nm.

A calibration curve was established using a monolithic capillary with non-modified epoxide groups (blank monolith). Standard solutions were prepared by mixing DPDS (1.19 M in acetonitrile) with different amounts of 2-mercaptoethanol (Sigma–Aldrich). The reaction was allowed to occur for at least 1 h at room temperature.

The break-through time of the non-modified capillary was determined using acetone in acetonitrile. Then the capillary with blank monolith was filled with the standard solution the same way as described for the thiol-modified capillaries, i.e. such that the solution did not reach the detection window. Subsequently, the capillary was flushed with acetonitrile for 35 min and the effluent was collected in a micro-vial. After addition of 200 μl acetonitrile the collected solution was transferred into an HPLC micro-vial and separated by RP-HPLC as described above.

A second external calibration curve was also constructed without using the poly(GMA–*co*-EDMA) blank monolith. The same standard solutions as used for the first calibration curve were prepared freshly by reaction of DPDS with 2-mercaptoethanol for 1 h. An aliquot of 7 μl was diluted with 200 μl acetonitrile and 20 μl of this solutions were injected into the HPLC system and separated analogous to the other solutions.

2.5. Removal of covalently linked 2-pyridylsulfide residues from the monolith surface

Before reduction the capillaries were preconditioned by rinsing with methanol–water (20:80 (v/v)) employing an HPLC pump. Solutions of tris(2-carboxyethyl) phosphine hydrochloride (TCEP, Fluka) were always freshly prepared by dissolving it in methanol–0.4 M aqueous sodium dihydrogen phosphate (20:80 (v/v)) in such a way that the reagent was present in a 50-fold molar excess with respect to determined thiol concentration on the monolith surface. The pH of the reagent solution was 4.5.

The 2-pyridylsulfide residues were removed by flushing the capillary with 50 μl of the TCEP solution employing a Hamilton syringe and a syringe pump followed by rinsing with methanol–water (20:80 (v/v)) for 1 h employing an HPLC pump. This procedure was repeated two or three times until all 2-pyridylsulfide residues were removed. This was checked by following the procedure described in Section 2.4.

2.6. In-column immobilization of t-BuCQN by radical addition reaction

A 0.25 M solution of *t*-BuCQN was prepared by dissolving it in methanol, and α,α′-azoisobutyronitrile (AIBN) (6 mg ml^{-1}, 0.037 M) was added as radical initiator. The mixture was sonicated for 5 min, filtered through a nylon membrane, and purged with nitrogen for 10 min. Then, the thiol-functionalised capillaries were flushed with 30 μl of the *t*-BuCQN solution using a Hamilton syringe and a syringe pump. The capillaries were sealed with GC septa and transferred to a water bath, where the radical addition of the chromatographic ligand occurred at 60 °C. After 24 h the capillaries were removed from the water bath, rinsed with methanol and then equilibrated with mobile phase using an HPLC pump.

2.7. Enantiomer separation with t-BuCQN-functionalised monolithic capillaries

(*R*,*S*)-DNB–Leu [1 mg ml^{-1} in mobile phase, containing 5-10 drops of acetone as electroosmotic flow electromotic flow (EOF) marker] was used as test substance. The mobile phase consisted of acetonitrile–methanol (80:20 (v/v)) containing 400 mM acetic acid and 4 mM triethylamine (both from Fluka). Injection was done electrokinetically by applying a voltage of −10 kV for 10 s. Electrochromatographic separations were carried out at a voltage of −10 kV, the temperature of the cassette containing the monolithic capillary column was 30 °C, and an external pressure of 6 bar was applied on inlet as well as outlet electrolyte vessel. The enantiomers were detected at a wavelength of 230 nm and acetone at 280 nm.

3. Results and discussion

3.1. Properties and reactivity of monolithic carrier

For the present study, a monolithic poly(GMA–*co*-EDMA), previously developed by Svec and Fréchet [8]

has been selected as carrier for the preparation of reactive thiol-modified monolithic capillary columns. The monolithic polymer was prepared from a polymerization mixture consisting of 24 (± 0.3)% (w/w) glycidyl methacrylate (GMA) as functional monomer, 16 (±0.3)% (w/w) ethylene dimethacrylate (EDMA) as crosslinker, 30 (±0.1)% (w/w) cyclohexanol and 30 (±0.2)% (w/w) 1-dodecanol as porogenic solvents. The polymerization was started by thermal initiation (at 60 °C) through decomposition of AIBN which was added at a concentration of 1% (w/w) related to the total amount of vinyl monomers. The porogenic solvents have been admixed in a ratio to adjust a modal pore diameter of the macroporous flow channels of about 1.1 μm (dry state) [43]. The pore volume of such a monolithic material is about 1.3 mL g^{-1} as measured by mercury intrusion porosimetry, and the specific surface area as determined by nitrogen adsorption–desorption isotherms is about 6 m^2 g^{-1} (calculated with the BET equation). The high crosslinking degree of 40% of the polymer effects a sufficient rigidity and stability of the monolithic polymer matrix which together with the large diameter of the flow-through pores assures a reasonably high permeability. This is of importance for the flow properties if the monolithic capillary is intended to be used in hydrodynamic mode, e.g. for capillary liquid chromatography, but facilitates also the rinsing with reagents and the washing steps of the monolith during the surface modification procedures. Yet, it does not necessarily represent the optimal pore diameter with regard to column efficiency for small molecules in capillary electrochromatography [14,21].

The poly(GMA–*co*-EDMA) monolith with above specified composition provides a sufficiently large number of reactive epoxide groups stemming from the 60 wt.% GMA in the polymerization mixture. These epoxy groups can be easily transformed to thiol groups by a nucleophilic substitution reaction with sodium-hydrogen sulfide according to the reaction scheme of Fig. 1. Assuming a complete incorporation of the GMA into the polymer and considering the density of the reaction mixture a 25 cm long monolithic capillary column of 100 μm i.d. should contain in total about 3.17 μmol epoxide groups (corresponding to about 4.23 mmol g^{-1} polymeric material).

The maximal thiol concentration on the surface of the monolithic support resulting after reaction with sodium-hydrogen sulfide will be, however, significantly lower than this theoretical number of total epoxy groups in the polymeric material. A number of factors can be made responsible for this non-quantitative transformation of epoxy groups to thiols. Most importantly, a significant portion of the epoxy groups are buried inside the polymer globules of the monolithic skeletons and cannot react. For example, Sykora et al. reported that 1.26 mmol g^{-1} of epoxy groups of above specified monolithic polymer have been transformed to weak anion-exchange functionalities with pure diethylamine in 3 h at 55 °C [44]. This corresponds to only about 29.9% of the total epoxy groups being modified. Since this number appeared to be much lower than the maximally accessible surface epoxy groups, we derivatized a poly(GMA–*co*-EDMA) monolithic material in a vial with excess of diethylamine choosing the conditions such (overnight at room temperature followed by 5 h at 60 °C) that all epoxy groups accessible on the surface are supposed to be derivatized. Elemental analysis (%N) performed on the carefully washed pieces of monolithic material revealed that about 75% of the epoxy groups were transformed. We believe that this percentage approaches the maximally accessible epoxy concentration, because a longer reaction did not yield a further increase of nitrogen in the material. Secondly, side reactions may occur inactivating a certain percentage of reactive epoxy groups. Since the thiol transformation reaction is carried out in aqueous media, as the main side reaction hydrolysis of the 2,3-epoxypropyl groups to inactive 2,3-dihydroxypropyl residues has to be considered [45], which therefore needs to be suppressed as much as possible (see above). Last but not least, the reaction itself possibly might not proceed quantitatively, in particular, if the reaction conditions are not fully optimized. To achieve satisfactory thiol modification and possibly be able to adjust the thiol concentration and thus the subsequent attachment of chromatographic moieties, we decided to optimize the modification reaction and to investigate the effect of various reaction parameters. We anticipated that for this purpose a method is required to measure the amount of reactive thiols on the monolith surface. Such a thiol quantification method had to be developed first.

3.2. Determination of thiol concentration on the monolith surface

The determination of the amount of reactive thiol groups generated by the nucleophilic substitution reaction with sodium-hydrogen sulfide is of particular importance for the control of the yield of the surface modification and a prerequisite or at least a helpful tool for a straightforward and effective optimization of the thiol modification reaction. We aimed at a direct chemical determination within the capillary rather than an external determination using polymeric material that was obtained from polymerization of the remainder of the reaction mixture followed by the same treatment with sodium-hydrogen sulfide. Reaction kinetics in the capillary is controlled by the diffusion of the reagents to the reactive sites in the macroporous and mesoporous channels, which may deviate significantly from the kinetics in a (stirred) vessel with (crashed) monolithic polymer as in the latter case. A systematic error cannot be excluded when results from determinations on remainder material are assumed to reflect exactly the surface concentration in capillaries.

For the determination of the concentration of thiol groups on the monolith surface the disulfide exchange reaction with 2,2′-dipyridyl disulfide (Fig. 2) was selected. The derivatization reaction was carried out in the CEC instrument.

The monolithic capillary with the thiol-modified porous polymer was filled with the DPDS solution hydrodynam-

ically (only over the length which contained the polymer plug) using the external pressurization system of the CEC instrument by applying a pressure of 8 bar. The required filling time was calculated from the break-through time of a void marker. After filling the capillary with the reagent the flow was stopped and the reaction according to scheme in Fig. 2 was allowed to proceed. It was assumed that the disulfide exchange reaction was complete within 1 h [40,46]. As reaction product, pyridine-2-thiol is formed in equimolar amount to the sulfhydryl groups on the monolith surface in the capillary, which is further converted to the tautomeric pyridine-2-thione form. It was claimed previously that the pyridine-2-thiol exists virtually only in the pyridine-2-thione form [40] which can conveniently be determined spectrophotometrically at its absorption maximum of 343 nm. After the reaction, the hydrodynamic flow was started again and the effluents containing the reaction product were collected and diluted (about 1:100). The final quantification was then carried out by RP-HPLC.

An external calibration curve was constructed with 2-mercaptoethanol as standard (in the range of 0.05 and 0.5 μmol, $n = 5$) and utilizing largely the same protocol. Instead of the thiol-monolith a plain non-modified poly(GMA–*co*-EDMA) capillary (blank monolith) was used and the DPDS and standard solutions were mixed before they were filled into the capillary. The following steps were performed as in the test with the thiol-monolith described above. The equation for the calibration curve was $y = 1.176(\pm0.037) \times 10^7 x - 0.016(\pm0.010) \times 10^7$, wherein y is the peak area of pyridine-2-thione as measured by RP-HPLC and x the thiol (2-mercaptoethanol) concentration in μmol. The correlation coefficient of $r = 0.9992$ indicates the high quality of the calibration function.

Two critical points were raised which could cause a systematic error: (i) the generated pyridine-2-thiol in the DPDS test and corresponding calibration is principally able to react with residual epoxide groups. (ii) The reaction product of the DPDS reaction might be adsorptively bound to the relatively lipophilic polymer surface by non-specific interaction. Acetonitrile as solvent should, however, minimize this risk. In both cases, the recovery of the reaction product would be lower than the equimolar concentration of surface-thiols.

To investigate whether either of these two complications occurs in the present test an alternative calibration curve in the same range with the equal number of calibration levels in absence of the blank monolith was set up. Thus, various concentrations of standard were reacted with excess of DPDS, diluted (1:100) and directly injected into the HPLC representing the calibration curve without polymer matrix effects. The equation for this calibration was $y = 1.169(\pm0.070) \times 10^7 x - 0.012(\pm0.018) \times 10^7$ and the correlation coefficient $r = 0.9973$. It is seen that both calibration curves agree fairly well. The typical deviation of the sulfhydryl concentrations on the thiol monolith surface calculated with the two distinct calibration functions was less than 1%. It was, therefore, concluded that no significant polymer matrix effect (non-specific adsorption) exists nor reaction of pyridine-2-thiol with residual epoxides occurs which both would falsify the results of our thiol-determination. The external calibration can henceforth be carried out directly with the second calibration curve without mimicking the presence of the monolith. The presented DPDS test was regarded as useful and served as basis for the optimization of the modification of the epoxide monoliths to a reactive thiol surface.

3.3. Optimization of epoxide opening reaction with hydrogen sulfide

In general, the modification proceeded less straightforward as expected from the previously reported reaction of the epoxide groups with diethylamine which was carried out in neat reactand. The solid character of sodium-hydrogen sulfide precludes in the present case the use of neat reactand. Initial experiments indicated the necessity for optimization of the reaction conditions for the transformation of the epoxy groups to terminal sulfhydryl moieties, because the overall yield in the aqueous–organic media turned out to be quite low. At this point it is emphasized that it is of utmost importance that the hydrogen sulfide solution is freshly prepared before use. For the tuning of this reaction, conditions were to be found which favor the bimolecular nucleophilic substitution (SN_2) reaction of the epoxide ring opening with hydrogen sulfide and concomitantly minimize competitive hydrolysis (ring opening of epoxide with hydroxide). Reaction time, pH, temperature, type and content of organic co-solvent, and concentration of hydrogen sulfide were considered as major influential variables and thoroughly investigated. Owing to the higher nucleophilicity of hydrogen sulfide over hydroxide, thiol formation should be favored over hydrolysis. However, a strong pH dependency may be expected. Thus, first the pH value of the reagent solution was optimized using 0.5 M sodium-hydrogen sulfide in methanol–0.1 M aqueous sodium dihydrogen phosphate (20:80 (v/v)). The effect of pH on conversion was examined in the pH range between 7.0 and 9.3.

The highest thiol concentrations on the monoliths were obtained with a sulfide solution of pH 8.14 (Fig. 3). In the monolithic capillary column which was treated with a reagent solution of such pH around 0.20 μmol 3-mercapto-2-hydroxypropyl residues were generated in total in the 25 cm long monolith, which is equivalent to a conversion of about 6.3% of all epoxide groups in the column.

At lower pH, a slightly lower thiol concentration was measured with the DPDS method, while at more alkaline pH the yield of surface-bound thiols was dramatically decreased. It seems that at more basic conditions the hydrolytic epoxide ring opening side reaction predominates. This is supported by a previous study on the pH-dependent hydrolysis of glycidylmethacrylate beads which revealed that the hydrolysis is of least significance close to neutral pH and becomes more important at more alkaline conditions [45].

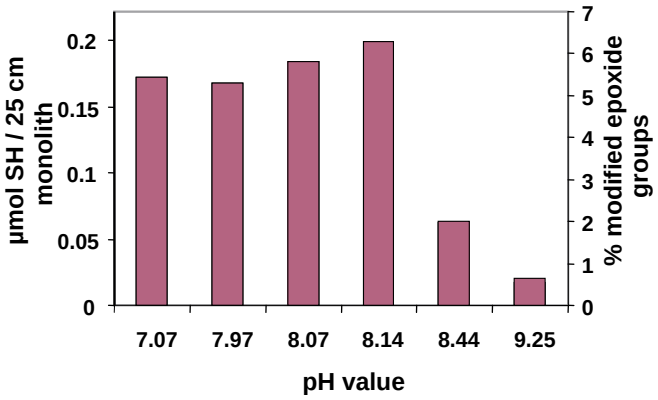

Fig. 3. Amount of thiol groups generated on the monolith surface at different pH values. Other reaction conditions: sodium-hydrogen sulfide was dissolved in methanol–0.1 M aqueous sodium dihydrogenphosphate (20:80 (v/v)) at a concentration of 0.5 mol l^{-1}, and the reaction time was 2 h at ambient temperature.

On the other hand, it might also be argued that other side reactions such as ring closure and formation of thiiranes could become relevant at such alkaline pH [47].

This transformation of oxiranes to thiiranes can be catalyzed thermally [47], a possible explanation why increase of reaction temperature did not lead to better results (Fig. 4). Another possible explanation is the kinetically favored sulfide over hydroxide attack which may be partly set off at higher temperatures.

In general, the conversion yields were quite poor in all cases (<6.3%). Since reactions on heterogeneous surfaces are known to occur with much slower kinetics it was attempted to improve the yield by raising the reaction time. Unfortunately, increase of reaction time brought no improvement with regard to the amount of thiol groups that were generated. The epoxide monolith which was reacted for 2 h contained as much thiol-residues as the one with 16 h reaction time (0.20 and 0.19 μmol/25 cm monolith, respectively).

Nucleophilic substitutions are strongly solvent dependent through its solvation effects of transition state and reactands.

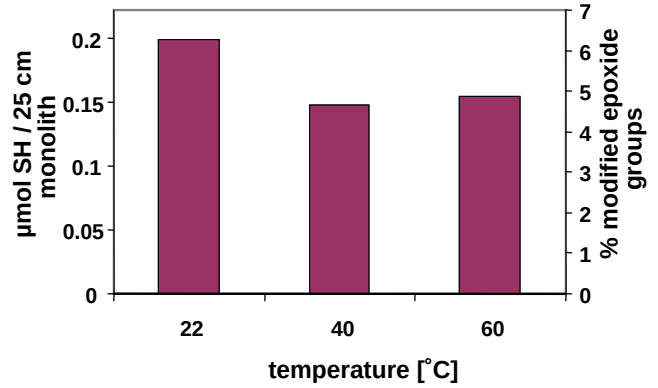

Fig. 4. Effect of temperature on the amount of thiol groups generated on the monolith surface. Conditions: Reagent solution and reaction time as in Fig. 3; pH: 8.15.

In the present case, aqueous-based media are required to keep the sodium-hydrogen sulfide reagent in solution and to avoid the formation of precipitates which would lead to clogging of the pores. However, an organic co-solvent was added to enhance the wettability of the lipophilic polymethacrylate backbone and pendent epoxypropyl groups. Acetonitrile and methanol were tested. With the latter higher surface-thiol concentrations were afforded. An increase of the methanol content from 20 to 40% (80 and 60% 0.1 M sodium dihydrogen phosphate buffer, respectively) resulted in a decrease of thiol residues from 0.20 μmol per 25 cm monolith to 0.14 μmol (concentration of sodium-hydrogen sulfide was 0.5 M, pH was 8.14).

According to the bimolecular reaction mechanism it can be deduced that the kinetics of the epoxide ring opening with sulfide will be enhanced when the sodium-hydrogen sulfide reagent concentration is increased. This acceleration in relation to the hydrolysis kinetics should consequently lead to increased conversion of epoxides to thiols which in turn is materialized by a higher surface coverage with pendent thiol groups. In fact, the measured thiol concentration of the monoliths grew proportionally when the concentration of the nucleophilic reagent was increased from 0.5 to 2 M (Fig. 5). The number of thiol-residues in the 25 cm monoliths could be raised from 0.20 μmol thiol groups (corresponding to 6.3% of all theoretical epoxide groups) to 0.54 μmol (16.9% of all epoxides). Further increase of sulfide concentration showed no improvement. The capillary which was treated with 3.5 mol of sulfide solution contained as much thiol groups as the capillary which was allowed to react with 1 molar solution (0.34 and 0.33 μmol SH/25 cm monolith, respectively). At higher concentrations sodium-hydrogen sulfide was no longer soluble under weakly basic conditions in the hydro-organic media.

A critical factor of in-column surface modification schemes is known to be the column-to-column repeatability.

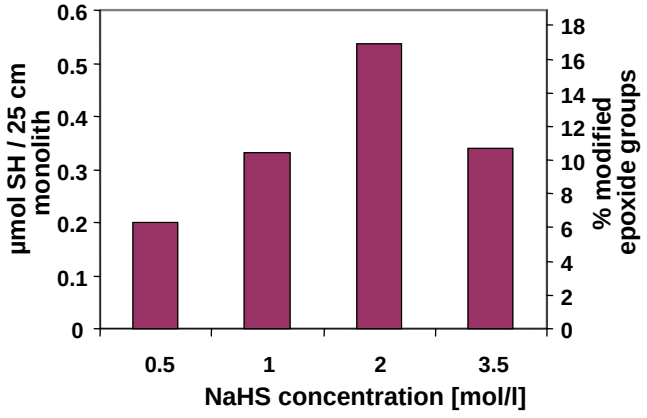

Fig. 5. Dependence of thiol concentration on the monolith surface on the concentration of sodium-hydrogen sulfide in the reagent solution. Conditions: The reagent was dissolved in methanol–0.1 M aqueous sodium dihydrogenphosphate (20:80 (v/v)), pH was 8.15, reaction time was 2 h at room temperature.

Hence, reproducibility of the optimized protocol was examined by treating three poly(GMA–*co*-EDMA) monolithic capillary columns, which were fabricated from the identical polymerization mixture, with 2 M sodium-hydrogen sulfide solution in methanol–0.1 M sodium dihydrogenphosphate (80:20 (v/v)), pH 8.15. Relative standard deviation for the determined thiol concentrations in the capillaries was calculated to be 3.21%. Considering that this value includes three distinct individual steps, namely column-to-column repeatability of monolith fabrication, repeatability of in-column epoxide transformation and repeatability of thiol quantification by the DPDS method the resultant reproducibility was assessed as highly satisfactory.

3.4. Reductive removal of 2-pyridyl sulfide residues from monolith surface after thiol quantification

The DPDS reaction for the quantification of the reactive thiol groups on the monolith surface modifies the sulfhydryl groups with 2-pyridyl sulfide residues (Fig. 2). The thiol groups are thus capped and cannot react in a subsequent functionalisation reaction such as radical addition. The advantage of the present assay for the determination of the thiol concentration is, however, the reversible nature of the thiol/disulfide exchange reaction. The monolith can be regenerated by a reductive removal of the 2-pyridyl sulfide residues, which reconstitutes the reactive sulfhydryl surface. A variety of common reducing agents has been investigated including 2-mercaptoethanol, dithiothreitol (DTT) and tris(2-carboxyethyl) phosphine hydrochloride (TCEP). Experiments with these three reducing agents showed that best results were obtained when TCEP was used (Fig. 6).

Both with DTT (0.1 M and 0.33 M in acetonitrile) and with neat 2-mercaptoethanol less than half of all thiol groups on the monolith surface could be regenerated, which means that only 30–50% of bonded 2-pyridyl sulfide residues could be removed in a first rinsing cycle (first reduction). Repeated flushing of the capillaries with these two reducing agents showed similar or even worse yields of reduced thiol groups (second and third reduction).

On the contrary, in case of TCEP 67% of original thiol groups could be regenerated after the first treatment. After flushing the capillary a second time with TCEP the reduction was complete. The amounts of reduced thiol groups of more than 100% with respect to the originally determined

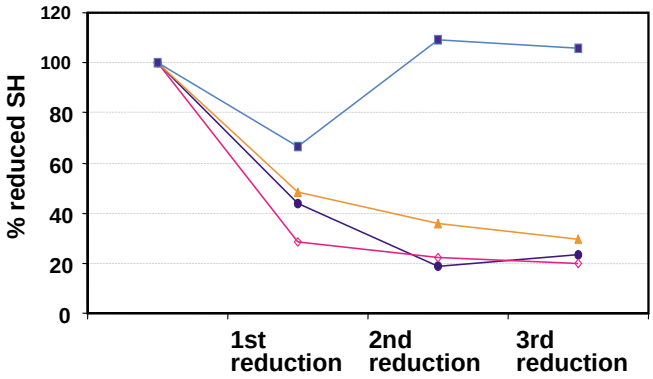

Fig. 6. Removal of 2-pyridylsulfide residues from the monolith surface using different reducing reagents. In case of dithiothreitol (DTT), (●) 0.1 M and (◇) 0.33 M, and (▲) neat 2-mercaptoethanol the capillaries were flushed with 60 μl of the reagent solution each, in case of (■) tris(2-carboxyethyl) phosphine hydrochloride (TCEP) 50 μl were used (which corresponds to 50 molar excess of reducing reagent).

sulfhydryl concentration are lying within the experimental error of the DPDS reaction method. An increase of the TCEP concentration in the reagent solution did not show a positive effect on the reduction yield in the first rinsing cycle. Repeated treatment with TCEP solution as reducing agent is therefore recommended.

3.5. Surface functionalisation by radical addition reaction

The usefulness of the reactive thiol monoliths for chromatographic purposes should be demonstrated by its functionalisation in a radical addition reaction with a quinine carbamate as a chromatographic model system.

Fig. 7 shows the reaction scheme of the immobilization of *t*-BuCQN on the monolith surface by radical addition. This chemistry, which is often selected as reference standard in our laboratory due to comparability causes, has been selected as model system for several reasons: (i) the sulfhydryl chemistry conveniently complies with typical standard immobilization procedures of this selector e.g. on thiolpropyl silica. (ii) The selector itself contains an ionizable group. The surface charge of the monolith will therefore be altered with the amount of selector moieties grafted to the surface. Hence, the strength of the EOF will also change with the surface concentration of the *t*-BuCQN selector which in turn depends on the concentration of reactive sulfhydryl available for the

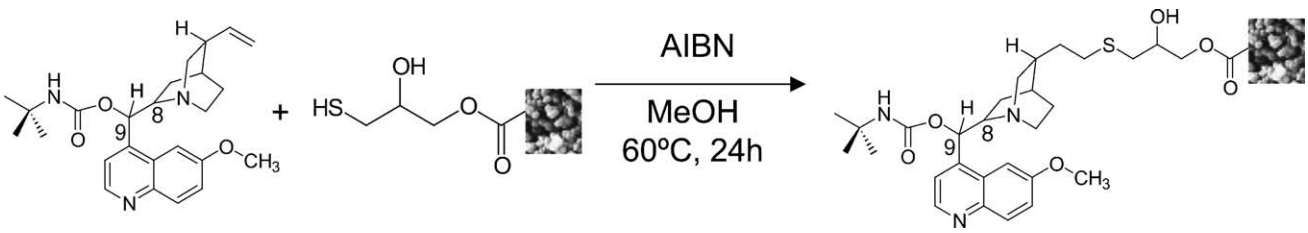

Fig. 7. Reaction scheme for the functionalisation reaction of the monolithic capillary columns by radical addition of *O*-9-*tert*-butylcarbamoylquinine (*t*-BuCQN) to the thiol groups.

radical addition. (iii) Through the use of an enantioselective chromatography as test system it is secured that separation is strictly relying on the chiral selector. Thus, enantioselectivity, i.e. separation of enantiomers will be solely obtained if a sufficient amount of chiral selector is available for intermolecular interaction with the solutes. (iv) Comparable data exist from a previous study that allow a comparison with in situ copolymerized monoliths obtained from a chiral monomer bearing similar functional chinchona carbamate moieties.

Overall, with this system the success of the present immobilization strategy and chemistry can conveniently be measured (electro)chromatographically by the EOF, retention factors, and enantioselectivity which should permit to assess the quality and usefulness of the proposed immobilization strategy via reactive thiol monoliths.

To show the dependency of the EOF on the concentration of thiol groups and thus in turn on the concentration of the chromatographic ligand on the monolith surface, several capillary columns containing different amounts of reactive thiol groups were modified with the chiral selector by identical treatment as described in Section 2.6. Assuming that the removal of the 2-pyridyl sulfide residues after determination of the thiol concentration on the monolith surface was complete, the capillary columns which were used for this series of experiments, all with a 25 cm monolithic segment contained 0.03 μmol thiol groups (0.8% of theoretical epoxide groups modified), 0.25 μmol (7.9%), 0.34 μmol (10.7%) and 0.44 μmol (13.9%). According to a differential DPDS determination before and after the selector immobilization, about 77.7% (±1.8) of all sulfhydryl-groups on the monolith surface could be modified with *t*-BuCQN or did not react anymore with the DPDS after the selector immobilization step (which corresponds to 2.34% R.S.D., $n = 3$), as was determined on three capillaries with identical thiol-concentration treated by the same procedures of selector binding. The plain poly(GMA–*co*-EDMA) blank monolith as well as the thiol-modified analogues do not carry groups on their surfaces that are ionized under given acidic CEC conditions and do therefore possess no measurable EOF or an extremely weak EOF. On the contrary, when the selector (*t*-BuCQN) is bonded to the monolith surface, which is positively charged under the conditions of the separation, the electroosmotic flow should become anodic and of reasonable strength.

Fig. 8a illustrates the dependency of the electroosmotic mobilities μ_{EOF} on the concentration of reactive thiol groups on the monolith surface (which is in turn materialized as different selector concentrations) in the four capillary columns. In Fig. 9a-d the corresponding electrochromatograms are shown.

In the first capillary (0.03 μmol thiol groups) the amount of positively charged selector bound on the monolith surface was obviously very low as no EOF could be determined within 160 min run time ($\mu_{EOF} < \pm 8.7 \times 10^{-6}\,cm^2\,V^{-1}\,s^{-1}$). The increase of thiol concentration on the monolith surface obviously also leads to enhanced selector concentration and surface charge σ, which translates into a higher electroosmotic flow [48].

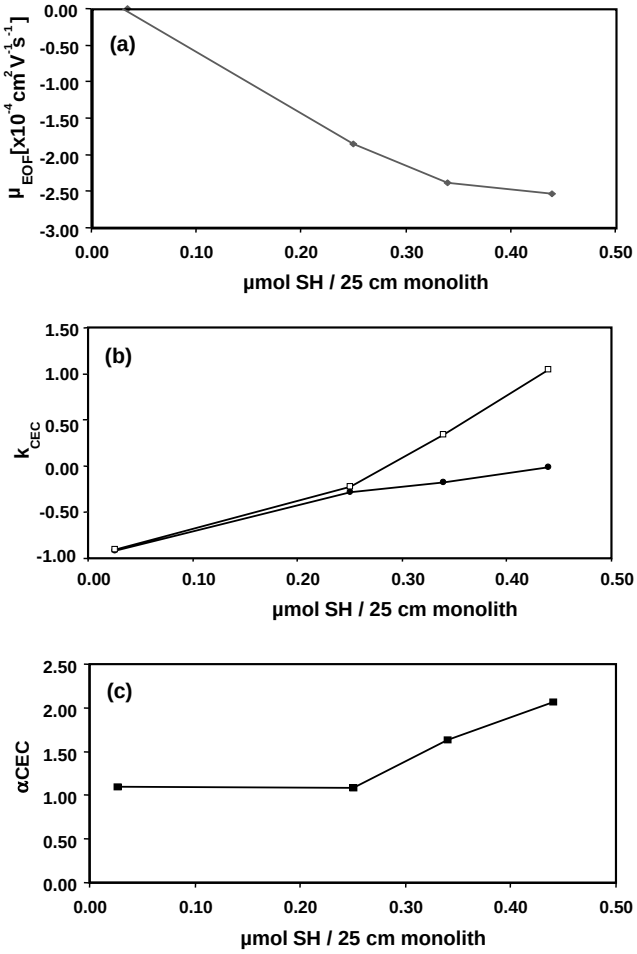

Fig. 8. Effect of thiol concentration on the monolith surface on: (a) the electroosmotic mobility (μ_{EOF}), (b) the retention factors (k_{CEC}) of (*R*,*S*)-DNB–Leu as calculated by $k_{CEC} = (t_R - t_0)/t_0$, (●) k_{CEC1}, (□) k_{CEC2}, and (c) the separation factors (α) as calculated by $\alpha = t_{R2}/t_{R1}$. EOF marker: acetone. For CEC conditions see Section 2.

Thus, the electroosmotic mobility could be amplified reaching $-1.85 \times 10^{-4}\,cm^2\,V^{-1}\,s^{-1}$ at 0.25 μmol SH/25 cm monolith (7.9% of theoretical epoxide groups), $-2.38 \times 10^{-4}\,cm^2\,V^{-1}\,s^{-1}$ at 0.34 μmol thiols (10.7%) and $-2.53 \times 10^{-4}\,cm^2\,V^{-1}\,s^{-1}$ at 0.44 μmol thiols (13.9%) with the utilized non-aqueous eluent.

Similarly, the increase in thiol and selector concentrations, respectively, should also be measurable by an increase of retention factors. At this point, it must be emphasized, however, that we are dealing with a dual separation system in which chromatographic (here anion-exchange) and electrophoretic mechanisms are mixed. Accordingly, the observed mobility, μ_{CEC}, of the analytes (DNB–Leu enantiomers) in the present anion-exchange CEC system depends on the electroosmotic mobility, μ_{EOF}, the effective electrophoretic mobility, μ_{ep}, of the negatively ionizable analytes and the purely chromatographic retention factor,

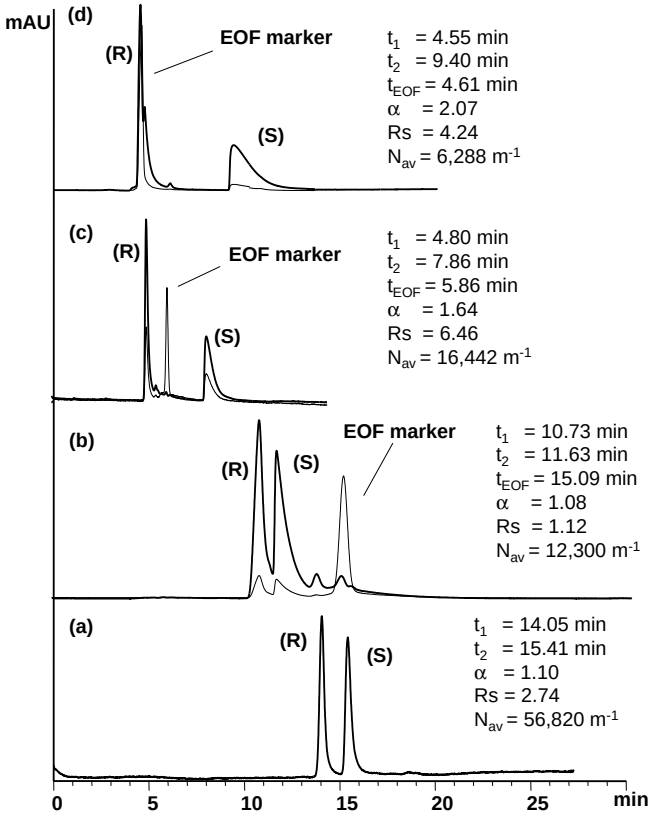

Fig. 9. CEC enantiomer separation of (*R*,*S*)-DNB–Leu on enantioselective monolithic capillary columns fabricated by surface functionalisation of monoliths carrying different amounts of reactive thiol groups by radical addition of *t*-BuCQN. (a) 0.03 μmol thiol/25 cm monolith (corresponding to 0.8% of epoxides or 0.04 mmol g^{-1} polymer), (b) 0.25 μmol thiol/25 cm monolith (7.9% of epoxides or 0.33 mmol g^{-1} polymer), (c) 0.34 μmol/25 cm monolith (10.7% epoxides or 0.45 mmol g^{-1} polymer), and (d) 0.44 μmol/25 cm monolith (13.9% of epoxides or 0.58 mmol g^{-1} polymer). For CEC conditions see Section 2. Bold lines: UV signal at 230 nm; plain lines: UV signal at 280 nm.

k_{LC}, on the chiral stationary phase as given by Eq. (1).

$$\mu_{CEC} = \frac{\mu_{EOF} + \mu_{ep}}{1 + k_{LC}} \quad (1)$$

Thus, the retention factor in CEC, k_{CEC}, as calculated by chromatographic terminology can be written as [49]:

$$k_{CEC} = k_{LC} - \mu_r k_{LC} - \mu_r \quad (2)$$

wherein the reduced mobility μ_r, which describes the relative contribution of electrophoretic mobility to the sum of both the driving forces μ_{ep} and μ_{EOF}, is defined as:

$$\mu_r = \frac{\mu_{ep}}{\mu_{ep} + \mu_{EOF}} \quad (3)$$

The retention window for the separation in CEC involving electrophoretic migration is spanned by:

$$-1 < k_{CEC} < \infty \quad (4)$$

Fig. 8b depicts the plot of k_{CEC} values versus the thiol concentration on the monolith. With the capillary having the lowest thiol and selector concentration, and thus negligible EOF, the retention factors of (*R*) and (*S*) enantiomers of DNB–Leu are approaching the limiting value of −1. It is evident that the electrophoretic migration contribution is the primary driving force for solute transport through the capillary column and chromatographic retention k_{LC} is weak. Nevertheless, this weak interaction is sufficient to afford enantioselectivity and baseline separation of the both enantiomers (Fig. 9a). As expected, the retention factors increase when higher amounts of thiol and hence also selector are grafted to the monolith surface. Still both DNB–Leu enantiomers elute before the EOF marker (k_{CEC} values <0), when a thiol concentration of 0.25 μmol per 25 cm monolith was determined by the DPDS assay (Fig. 9b). On the contrary, the retention factors of the stronger retained (*S*)-enantiomer reached positive values with a thiol concentration of 0.34 and 0.44 μmol/25 cm column (see Fig. 8b as well as Fig. 9c and d). This is indicative for a chromatographic dominance of the separation in these cases. In contrast, the weaker interacting (*R*)-enantiomer was still eluted before the EOF marker on both columns with higher thiol and selector loadings.

As separation factors calculated from k_{CEC} are without (thermodynamic) meaning, we use here the ratio of the retention times of second and first eluted enantiomer as separation factor ($\alpha = t_{R2}/t_{R1}$) to describe the dependence of the enantioselectivity on the thiol and selector concentration, respectively. Again, the increase in the thiol and selector concentration is paralleled by an increase of the separation factors (Fig. 8c), as was expected from the reliance of enantioselectivity on the chromatographic process which becomes more dominating at elevated thiol and selector concentrations. The overall trend is plausible and convincing. It indicates that indeed the amount of selector grafted onto the monolith surface is depending on the number of thiols on the monolithic substrate available for covalent attaching of the selector. It also demonstrates that by the present immobilization approach a sufficient number of chromatographic moieties can be bonded to the monolith surface by the post-synthesis modification procedure.

If the performance and separation capability of the enantioselective monolithic column shown in Fig. 9d is compared with an analogue obtained by the in situ approach [14] (Fig. 10), it turns out that the post-synthesis modification procedure is quite competitive. In this context, however, it needs to be noted that the chiral selector of the in situ prepared monolith is not exactly the same as in the post-modification approach. Minor structural differences such as a switched configuration at C8/C9 (quinine versus quinidine configurations i.e. pseudo-enantiomeric selectors) and different carbamate residues (*tert*-butyl versus quasi-ethyl carbamate) make the comparison somehow laming, since it could be argued that these differences exert a significant effect on separation factors. In a previous study, however, in which both types of selectors were in situ polymerized, it turned out that the separation factors

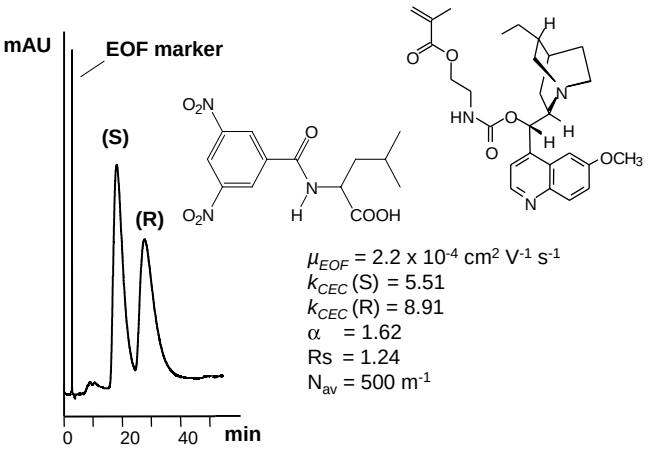

Fig. 10. CEC enantiomer separation of (*R,S*)-DNB–Leu on enantioselective monolithic capillary columns fabricated by in situ copolymerization of chiral monomer (8% (w/w)), GMA (16% (w/w)) and EDMA (16% (w/w)) in the presence of cyclohexanol (44% (w/w)) and 1-dodecanol (16% (w/w)) [14]. UV polymerization for 16 h at room temperature. Mode pore diameter, 1 μm. For CEC conditions see Section 2.

were only moderately higher for the chiral cinchona alkaloid monomer with *tert*-butylcarbamate residue [16]. The comparison with the separation in Fig. 10 is, therefore, a reasonable first guess to assess the quality of the post-modification approach. In the in situ polymerized monolith the selector content amounted to 20 wt.% of the polymer and by elemental analysis a selector incorporation of 0.6 mmol g^{-1} polymer was determined, certainly not all of it chromatographically available on the surface. The electroosmotic mobility of this in situ monolith was determined to be -2.2×10^{-4} cm^2 V^{-1} s^{-1} compared to -2.53×10^{-4} cm^2 V^{-1} s^{-1} of the post-functionalised monolith (0.42 mmol thiols g^{-1} polymer). The higher selector content of the in situ polymerized monolith explains the stronger retention as compared to the post-functionalised analogue.

Unfortunately, the efficiencies of the separation shown in Fig. 9d are clearly below what may be expected and has been achieved in CEC (e.g. up to 250,000 plates m^{-1} with in situ prepared quinidine carbamate monoliths [15]). They further decrease with the increase in selector concentration (Fig. 9b–d). These moderate plate numbers are partly due to a macropore diameter that is too large for small molecules. A further optimization of the monolithic capillaries with focus on efficiencies, which was beyond the scope of the present work, should therefore address the pore size that is expected to show an optimum at a modal pore diameter of about 600 nm [14]. Reducing the crosslinking to about 20% (w/w) EDMA is another option for improving the performance of the monoliths. Moreover, it needs to be emphasized that much higher plate counts and also enhanced selectivities have been obtained previously with more hydrophilic 2-hydroxyethylmethacrylate-based in situ polymerized cinchona alkaloid monoliths than GMA-based analogs, which seemed to exhibit detrimental non-specific interactions [14–16]. Hydrolysis of the residual epoxy groups of the monolith as previously suggested by Peters et al. [50] or hydrophilic end-capping of non-modified thiol groups could help to further improve the performance of the monolithic capillaries. Both, however, require additional steps which would make the entire procedure of the column fabrication lengthy and inconvenient. This limitation of the GMA-based monoliths may impose some restrictions on their use in practical applications of enantiomer separation. The moderate plate numbers however would not be a serious problem for our intended purpose, in which the thiol-modified monolithic capillaries are to be utilized for the selector screening. Straightforward immobilization by rinsing several capillaries in parallel and consumption of low selector quantities makes it an ideal approach to find out the most promising candidate that yields the highest separation factor in a set of structural analogs.

Finally the reproducibility of the whole procedure of preparing surface functionalised monoliths including the radical addition of *t*-BuCQN was determined by treating three capillaries analogously. The relative standard deviations for the retention times of the enantiomers and the EOF marker were 3.21% for the (*R*)-enantiomer (the mean retention time was 4.42 ± 0.14 min), 13.50% for the (*S*)-enantiomer (7.89 ± 1.07 min) and 5.99% for acetone (4.71 ± 0.28 min). Considering the multiplicity of the steps involved these values appear quite satisfactory. Eventually, it makes the methodology worthwhile to be applied in cases where straightforward in situ polymerization is beyond consideration.

4. Conclusion

This paper describes the preparation of thiol-modified poly(glycidyl methacrylate–*co*-ethylene dimethacrylate) monoliths by transformation of the epoxide groups of the polymer stemming from the GMA monomer by nucleophilic substitution reaction with sodium-hydrogen sulfide. This surface modification reaction was systematically optimized so that the yield of modified epoxide groups could be increased from <0.5%, which were obtained by the first non-optimized experiments, up to 17% (corresponding to 0.71 mmol thiols g^{-1} polymer). The amount of reactive thiol groups generated on the monolith surface could be determined directly in the capillary columns in a disulfide-exchange reaction using 2,2′-dipyridyl disulfide.

In a second step, it was possible to functionalize the monoliths with a chromatographic ligand which was attached to the surface by radical addition reaction. The applicability of these functionalised monolithic capillary columns for CEC could be demonstrated by the enantiomer separation of (*R,S*)-DNB–Leucine with good enantioselectivity and a suitable electroosmotic flow.

Finally, it can be concluded that the presented methodology appears to be an interesting and straightforward alter-

native to other monolith functionalisation strategies, in particular due to its great chemical flexibility and controlled surface chemistry.

Acknowledgements

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References

[1] Z. Deyl, F. Svec (Eds.), Capillary Electrochromatography, Elsevier, Amsterdam, 2001.
[2] E.F. Hilder, F. Svec, J.M.J. Fréchet, Electrophoresis 23 (2002) 3934.
[3] M. Lämmerhofer, W. Lindner, in: F. Svec, T.B. Tennikova, Z. Deyl (Eds.), Monolithic Materials, Elsevier, Amsterdam, 2003, p. 489.
[4] N. Ishizuka, H. Minakuchi, K. Nakanishi, N. Soga, H. Nagayama, K. Hosoya, N. Tanaka, Anal. Chem. 72 (2000) 1275.
[5] M. Petro, F. Svec, J.M. Fréchet, J. Chromatogr. A 752 (1996) 59.
[6] I. Gusev, X. Huang, Cs. Horvath, J. Chromatogr. A 855 (1999) 273.
[7] A. Premstaller, H. Oberacher, C.G. Huber, Anal. Chem. 72 (2000) 4386.
[8] F. Svec, J.M.J. Fréchet, Anal. Chem. 64 (1992) 820.
[9] F. Svec, J.M.J. Fréchet, Ind. Eng. Chem. Res. 38 (1999) 34.
[10] S. Hjertén, Ind. Eng. Chem. Res. 38 (1999) 1205.
[11] A. Palm, M.V. Novotny, Anal. Chem. 69 (1997) 4499.
[12] A. Maruska, in: F. Svec, T.B. Tennikova, Z. Deyl (Eds.), Monolithic Materials, Elsevier, Amsterdam, 2003, p. 143.
[13] M. Lämmerhofer, F. Svec, J.M.J. Fréchet, W. Lindner, J. Chromatogr. A 925 (2001) 265.
[14] M. Lämmerhofer, E.C. Peters, C. Yu, F. Svec, J.M.J. Fréchet, W. Lindner, Anal. Chem. 72 (2000) 4614.
[15] M. Lämmerhofer, F. Svec, J.M.J. Fréchet, W. Lindner, Anal. Chem. 72 (2000) 4623.
[16] M. Lämmerhofer, E. Tobler, E. Zarbl, W. Lindner, F. Svec, J.M.J. Fréchet, Electrophoresis 24 (2003) 2986.
[17] M.G. Schmid, N. Grobuschek, C. Tuscher, G. Gübitz, A. Vegvari, E. Machtejevas, A. Maruska, S. Hjertén, Electrophoresis 21 (2000) 3141.
[18] T. Koide, K. Ueno, J. High Resolut. Chromatogr. 23 (2000) 59.
[19] T. Koide, K. Ueno, J. Chromatogr. A 909 (2001) 305.
[20] R. Hahn, A. Podgornik, M. Merhar, E. Schallaun, A. Jungbauer, Anal. Chem. 73 (2001) 5126.
[21] E.C. Peters, M. Petro, F. Svec, J.M.J. Fréchet, Anal. Chem. 69 (1997) 3646.
[22] Z. Chen, T. Hobo, Anal. Chem. 73 (2001) 3348.
[23] J. Kang, D. Wistuba, V. Schurig, Electrophoresis 23 (2002) 1116.
[24] B. Chankvetadze, C. Yamamoto, Y. Okamoto, Chem. Lett. 32 (2003) 850.
[25] F. Svec, J.M.J. Fréchet, J. Chromatogr. A 702 (1995) 89.
[26] S. Zhang, X. Huang, J. Zhang, Cs. Horvath, J. Chromatogr. A 887 (2000) 465.
[27] Q. Luo, H. Zou, X. Xiao, Z. Guo, L. Kong, X. Mao, J. Chromatogr. A 926 (2001) 255.
[28] O. Kornysova, P.K. Owens, A. Maruska, Electrophoresis 22 (2001) 3335.
[29] M. Petro, F. Svec, J.M.J. Fréchet, Biotechnol. Bioeng. 49 (1996) 355.
[30] S. Xie, F. Svec, J.M.J. Fréchet, Biotechnol. Bioeng. 62 (1999) 30.
[31] L.G. Berruex, R. Freitag, T.B. Tennikova, J. Pharm. Biomed. Anal. 24 (2000) 95.
[32] M. Vodopivec, A. Podgornik, M. Berovic, A. Strancar, J. Chromatogr. B 795 (2003) 105.
[33] C. Viklund, F. Svec, J.M.J. Fréchet, Biotechnol. Bioeng. 13 (1997) 597.
[34] E.C. Peters, F. Svec, J.M.J. Fréchet, Adv. Mater. 9 (1997) 630.
[35] T. Rohr, E.F. Hilder, J.J. Donovan, F. Svec, J.M.J. Fréchet, Macromolecules 36 (2003) 1677.
[36] D.S. Peterson, T. Rohr, F. Svec, J.M.J. Fréchet, Anal. Chem. 75 (2003) 5328.
[37] U. Meyer, F. Svec, J.M.J. Fréchet, C.J. Hawker, K. Irgum, Macromolecules 33 (2000) 7769.
[38] F.M. Sinner, M.R. Buchmeiser, Angew. Chem., Int. Ed. Engl. 39 (2000) 1433.
[39] D.R. Grassetti, J.F. Murray, Arch. Biochem. Biophys. 119 (1967) 41.
[40] M.R.F. Ashworth, The Determination of Sulphur-Containing Groups, vol. 2, Analytical Methods for Thiol Groups, Academic Press, London, 1976.
[41] P. Sevcikova, Z. Glatz, J. Tomandl, J. Chromatogr. A 990 (2003) 197.
[42] J.A. Burns, J.C. Butler, J. Moran, G.M. Whitesides, J. Org. Chem. 56 (1991) 2648.
[43] C. Viklund, F. Svec, J.M.J. Fréchet, K. Irgum, Chem. Mater. 8 (1996) 744.
[44] D. Sykora, F. Svec, J.M.J. Fréchet, J. Chromatogr. A 852 (1999) 297.
[45] H. Hrudkova, F. Svec, J. Kalal, Br. Polym. J. (1977) 238.
[46] R. Nogueira, N.M. Maier, M. Lämmerhofer, W. Lindner, Anal. Chim. Acta, in preparation.
[47] K.H. Büchel (Ed.). Houben-Weyl, Methods of Organic Chemistry, vol. E11 (Part 2). Thieme, Stuttgart, 1985, p. 1493.
[48] M.M. Dittmann, G.P. Rozing, J. Chromatogr. A 744 (1996) 63.
[49] M.M. Dittmann, K. Masuch, G.P. Rozing, J. Chromatogr. A 887 (2000) 209.
[50] E.C. Peters, K. Lewandowski, M. Petro, F. Svec, J.M.J. Fréchet, Anal. Comm. 35 (1998) 83.