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Polyrotaxane approach for synthesis of continuous beds for capillary electrochromatography

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Abstract

The polyrotaxane formation approach was evaluated for synthesis of continuous beds for capillary electrochromatography. This approach has the advantage of generating diverse electroosmotic and chromatographic properties without chemical reactions. The polyrotaxane derivatized continuous beds were formed adding the macrocyclic compounds to the solution of neutral acrylic monomers and crosslinker prior to the initiation of the polymerisation. Cationic and anionic derivatives of β-cyclodextrin were used as macrocyclic compounds. Investigation of the electroosmotic properties indicated a template directed and enthalpy controlled self-assembly of the polyrotaxanes during the polymerisation of the continuous beds. This process was monomer-composition dependent and favored by the hydrophobicity of the polymeric skeleton. The morphology of the continuous beds was evaluated using high-resolution optical microscopy with CCD camera and atomic force microscopy. Reversed-phase capillary chromatography driven by electroosmosis, originating from the polyrotaxane structure, was performed using several test mixtures. Not primarily designed for the chiral chromatography the polyrotaxane derivatized continuous beds demonstrated enantioselective separation of D,L-metoprolol. The stability of the polyrotaxane derivatized continuous beds was tested. The beds demonstrated reproducible electroosmotic properties in the range from pH 4 to pH 9 (RSD=0.69%).

Keywords: Continuous bed; Electrochromatography; Stationary phases, electrochromatography; Enantiomer separation; Rotaxanes

1. Introduction

The continuous bed (sometimes referred to as monolith) technique is a mature approach to prepare chromatographic columns by in situ synthesis of nonparticulate stationary phases, which was proposed and developed approximately 10 years ago [1–3]. This approach now covers many chromatographic separation modes [4,5]. Continuous beds may be regarded as fourth generation of the bioadsorbents after the earlier synthesized polysaccharide-based, cross-linked and coated and monodisperse materials [6]. Nevertheless, continuous beds are successfully applied for separation of small organic molecules as well [4,5]. Different types of the polymers such as acrylic [7,8], styrene–divinylbenzene [9,10], polynorbornene [11] and silica-based

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stationary phases [12,13] are used as continuous bed packing materials for HPLC and capillary format separations.

Rotaxanes are molecular species, in which a macrocyclic molecule is threaded on to a linear one with bulky blocking groups at both ends (Fig. 1). Recently, a number of protocols based on self-assembly have been developed for the synthesis of these mechanically-interlocked compounds. Different macrocyclic compounds such as cyclodextrins, crown ethers, calixarenes can be employed for the synthesis of rotaxanes.

Polyrotaxanes are polymeric structures that contain several rotaxane moieties. According to the structural design polyrotaxanes can be classified as those with rotaxane moieties in the main chain or the side chains [14–16]. Thermodynamically, the self-assembly of the rotaxanes occurs due to electrostatic or hydrophobic interaction, and results in a negative enthalpy change (ΔH) or entropic term (ΔS) driven threading, when macrocyclic molecules are mixed with linear ones. The entropic term driven process is less efficient and threading yield is statistically dependent on the concentration of the cyclic and linear components. As shown by Gibson's group [17], the yield of polyrotaxanes formed for the same crown ether as a host molecule at entropy driven threading is 30.3 times less compared to enthalpy driven process, when the ratios of the cyclic component per axial molecule monomer unit (n/m) are compared.

α-, β- and γ-Cyclodextrins are cyclic oligomers of amylose consisting of six, seven or eight glucose units, respectively. The inclusion of polymers by cyclodextrins is of growing interest as it gives the possibility to change the physical and chemical properties of the polymers without the necessity of polymer analogous reactions. The different properties of rotaxanes compound to those of common polymers are caused by the flexible and movable connections of the cyclic and the linear components. Due to their unique characteristics the rotaxanes are used in development of the nanoscale devices, such as molecular shuttles, switches and information-storage systems [18]. Many synthetic procedures for cyclodextrin based rotaxanes and polyrotaxanes are developed by Wenz's group [19–21]. Harada reported the synthesis of molecular nanotubes based on the assembly and crosslinking of cyclodextrin polyrotaxane on the poly(ethylene glycol) chain [22]. In 1975 Callahan et al. [23] published an article upon the network structured polymer obtained by crosslinking the polybutadiene threaded 36-membered oligopeptide (valinomycin) macrocycles. Several articles describing crosslinked polyrotaxanes were published in the mid 90s of the last century. Garrido et al. [24,25] and Clarson et al. [26] have shown that a polyrotaxane gel can be formed from relatively large cyclics of poly(dimethylsiloxane) by threading them with linear chains of the same polymer and crosslinking the ends of linear chains with tetrafunctional or bifunctional crosslinkers. Recently Okumura and Ito [27] described the three-dimensional gel structure of an α-cyclodextrin and poly(ethyleneglycol) based polyrotaxane, covalently crosslinking cyclodextrin rings.

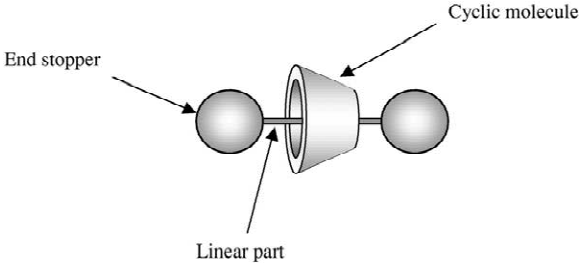

Fig. 1. Supramolecular structure of rotaxane.

1.1. The aim

In this study we focussed on the potential use of rotaxanes for the synthesis of continuous beds for capillary electrochromatography (CEC) from acrylamide comonomers in the presence of β-cyclodextrin derivatives.

2. Materials and methods

2.1. Materials

Ammonium persulfate (APS) and *N*,*N*,*N*′,*N*′-tetramethylethylenediamine (TEMED) were of analytical grade from Reanal (Budapest, Hungary). Piperazine diacrylamide (PDA), methacrylamide (MA) and vinylsulfonic acid (VSA) were purchased from Fluka

(Buchs, Switzerland). Methanol (MeOH), acetonitrile (MeCN) of high-performance liquid chromatography (HPLC) grade, glacial acetic acid (HOAc), triethylamine (TEA), methacryloxypropyl trimethoxysilane (Bind Silane A 174) and ammonium sulfate were bought from E. Merck (Darmstadt, Germany). *N*-isopropyl acrylamide (IPA), sulfated β-cyclodextrin containing 7–11 sulfo groups (sulfated β-CD) and neutral β-CD were purchased from Aldrich (Milwaukee, WI, USA). 6-amino β-CD was from Advanced Separation Technologies (USA). Racemic metoprolol was obtained from Medical Chemistry, AstraZeneca R&D Mölndal (Mölndal, Sweden). Fused silica capillary (50 μm I.D.×375 μm O.D.) was obtained from Polymicro Technologies (Phoenix, USA).

2.2. Apparatus

All chromatographic experiments were carried out using in-laboratory built capillary electrochromatography apparatus consisted of a Model UVM II detector (Pharmacia, Sweden) or Linear UVIS 200 (Reno, NV, USA) modified by installing ball lenses for on-capillary detection and a Model DA-30 high voltage source (SpectroVision, USA) or high voltage source PALM 897 designed by Per-Axel Lidström at Uppsala University. The continuous beds were washed and conditioned using an HPLC pump at constant pressure (12 MPa).

2.3. Preparation of capillary columns

The fused-silica capillaries were pretreated with Bind Silane A 174 in order to attach covalently the methacryloyl groups to the capillary inner wall, as described previously [28]. The general procedure for the preparation of different polyrotaxane-modified continuous beds was as follows (if not indicated elsewhere): 100 mg of β-CD derivative, 125 mg of PDA, 75 mg of MA, 100 mg of IPA and 64 mg of ammonium sulfate were dissolved in 1 ml of 50 m*M* sodium phosphate buffer (pH 7.0). The reaction mixture was degassed by vacuum and then 40 μl of a 10% (v/v) aqueous solution of TEMED and 40 μl of a 10% (w/v) aqueous solution of APS were added. The polymerisation mixture was immediately sucked into the capillaries, using a water jet pump. The polymerisation, which started within a couple of minutes, was left to proceed overnight. The next day the formed continuous beds were washed with distilled water and running buffer.

2.4. Microscopic investigations

A microscope (MMP-4, LOMO, Russia) equipped with a high-resolution, high-sensitivity CCD B/W camera KAM 02 was coupled to a personal computer via Data Translation unit DT 3152 to obtain micrographs. Three-dimensional micrographs were obtained using Image-Pro Plus v 4.1 software with programmable x, y and z stage. The optical sectioning method was applied to determine the location of each point on the surface. The surface morphology image was investigated with an Atomic Force Microscope (Q-Scope 250, Quesant, CA, USA). A triangular V-shaped Si3N4 cantilever with static stiffness 0.16 Nm^{-1} and the geometry $L=180$ μm, $a=25$ μm and $b=0.8$ μm (as reported by manufacturer) was used. Atomic force microscope was operated in the contact mode.

3. Results and discussion

3.1. Morphology of the continous beds

The synthesis of polyrotaxane-derivatized continuous beds is based on in situ polymerisation of selected comonomers and crosslinker in the presence of macrocyclic molecules. Cationic and anionic polyrotaxane modified acrylic continuous beds were synthesized in the Bind Silane A174 pretreated fused-silica capillaries of 50 μm I.D. Sulfated β-CD was used as macrocyclic compound in order to form negatively charged polyrotaxanes. Positively charged polyrotaxanes were formed with 6-amino β-CD. The total concentration of the comonomers (%T—concentration of comonomers plus crosslinker %, w/v) was 21.4%. The crosslinker concentration (%C—crosslinker fraction in the mixture of comonomers plus crosslinker, %) in the comonomer mixture was 41.6%.

One of the essential differences of the continuous bed technique comparing to conventional particulate stationary phases is that the synthesis of the station-

ary phase is accomplished simultaneously with the packing of the column, which means that the morphology of the bed is formed during the synthesis of the stationary phase. Since the resolution of the continuous beds is closely related to their morphological characteristics the morphology of the packing is of utmost importance in order to obtain successful separations. Therefore, it is important to investigate and control the morphology-governing parameters during the synthesis of the bed. Earlier we have shown that high resolution optical microscopy complementary to other microscopic methods might be a useful and convenient means for a simple and fast evaluation of the continuous bed morphology [29]. It does not require additional sample pretreatment or special preparation and provides a possibility for in-situ inspection of the bed homogeneity across the capillary length. In Fig. 2 are shown optical three-dimensional micrographs obtained for the rotaxane modified continuous beds formed at different ionic strengths of the polymerisation media. From the micrographs it is obvious that the increase of the salt concentration (0.016–0.08 g/ml of ammonium sulfate) results in a higher roughness of the continuous bed surface, indicating bigger channels in the polymeric skeleton and better permeability of the bed. Less uniform polymeric skeleton of the continuous bed increases the first term in the van Deemter equation. Broad channels obtained at higher ionic strength increase the resistance to the mass transfer in the mobile phase. The third term of the van Deemter equation may increase due to the smaller surface area and the thicker walls of the polymeric skeleton, assuming that the skeleton is porous [30]. High permeability (low back-pressure) is a decisive factor of the pressure driven chromatography, although not a limiting factor in CEC. As recently shown by Palm and Novotny [31] the continuous beds can be electroosmotically rinsed and conditioned after the synthesis. Electroosmotic conditioning was applied also by Hjertén et al. [32] and Fujimoto [33] for conditioning of low concentration homogeneous gels for CEC. On the other hand it is very convenient to have not too high back-pressure of the continuous bed to flush the capillary by means of HPLC pump, if bubbles form during the electrochromatographic run. From this point of view, the beds formed at a concentration of ammonium sulfate of 0.048 g/ml (Fig. 2b) were optimal to obtain a high efficiency-to-permeability ratio. The pressure drop for these beds was 0.23 MPa/cm at a linear velocity of the aqueous mobile phase of 2.23 cm/min.

The rapid development of the probe microscopy methods and instrumentation during the last two decades provided researchers with powerful tools for direct investigation of various surfaces in the nanometer scale range. We used contact mode and intermittent contact AFM for the phase imaging to investigate the rotaxane derivatized continuous beds and, thus, to evaluate the polymeric skeleton. The samples were dried for AFM experiments. From previous microscopic investigations we know, that some shrinkage and narrowing of the perfusive channels occurs when continuous bed is dried. The results, therefore, should be interpreted taking in account these changes, especially when absolute dimensions of the pores are compared between different microscopic methods [29]. The AFM image of the continuous bed, formed at high ionic strength (concentration of ammonium sulfate 1.25 g/ml, Fig. 3), demonstrates globularly structured surface, which is built of fused together roundly shaped polymeric fragments of 65–115 nm and pores or cavities ranging from 23 nm till 60 nm and more. The total volume fraction of the pores in the polymer is around 20%. The resolution of the images was too low to evaluate mesopores or micropores. AFM investigation of the larger area (10×10 μm) revealed channels of 1.5–4 μm diameter present in the polymeric skeleton, which is consistent with optical microscopy data. The channels larger than 800 nm are referred to as perfusive. They permit liquid flow under the pressure gradient, as do the 1.5–4 μm large pores.

3.2. Generation of electroosmotic flow via rotaxanes

Previously we have shown, that electroosmosis may be induced and direction of the electroosmotic flow may be reversed by forming the polyrotaxane modified continuous beds with cationic or anionic derivatives of β-CD [34]. In this study, different compositions of the polymerisation mixture were used to form polyrotaxane modified continuous beds and investigate their electroosmotic properties (Fig.

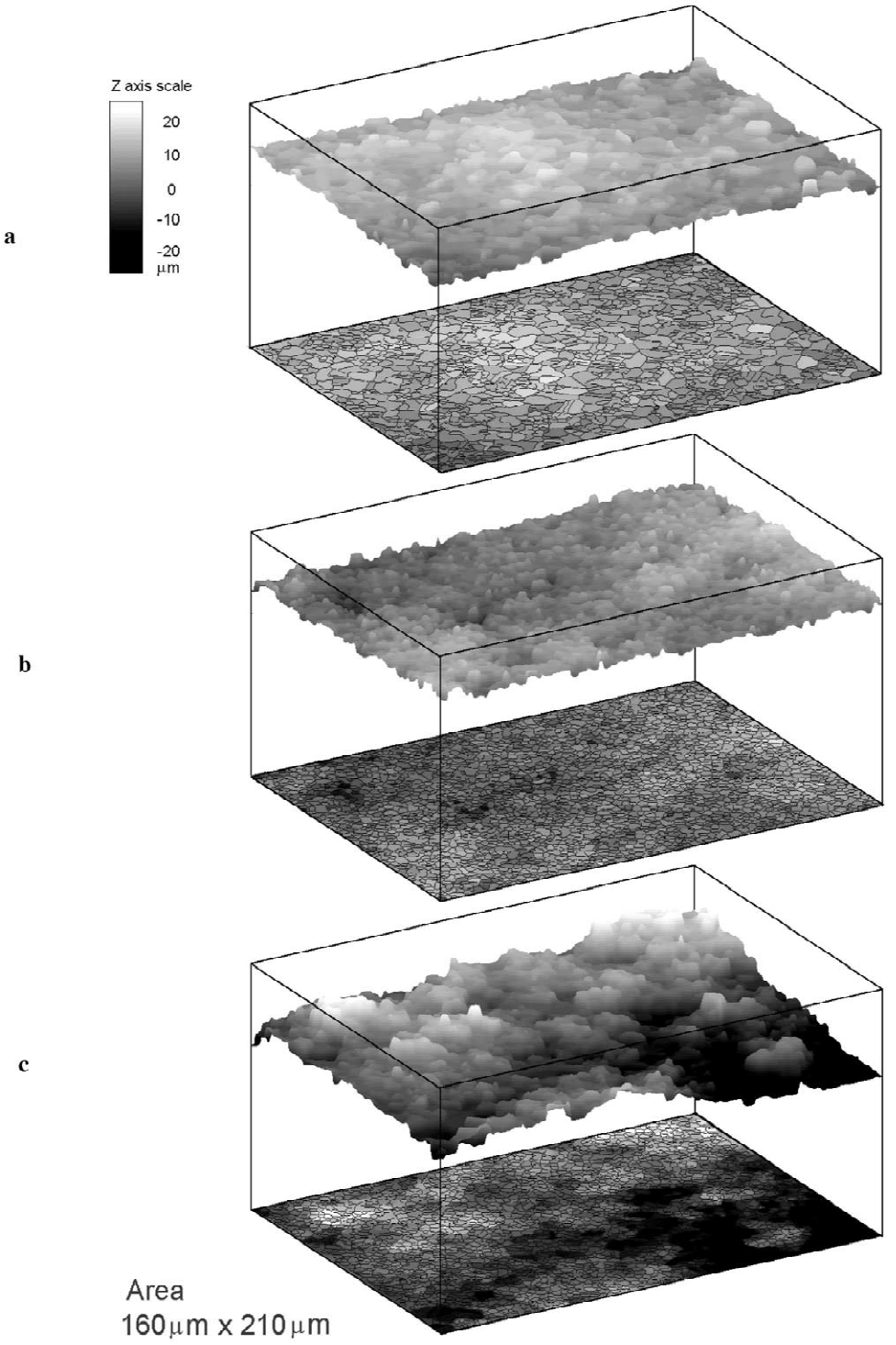

Fig. 2. Optical images of the polyrotaxane derivatized continuous beds, formed at different ionic strengths. Ammonium sulfate concentration: (a) 0.016 g/ml; (b) 0.048 g/ml; (c) 0.08 g/ml.

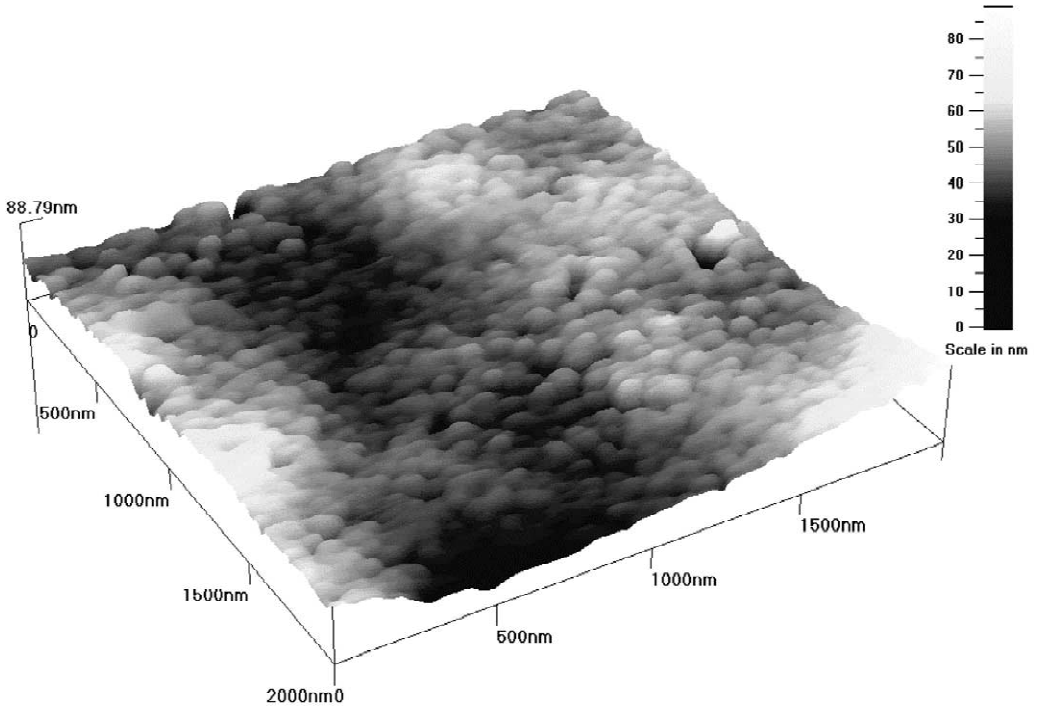

Fig. 3. AFM image of continuous bed formed at high ionic strength.

4). Influence of the hydrophobicity of the polymeric skeleton on polyrotaxane formation was evaluated assuming, that template-directed threading occurs and it is mainly based on the nonpolar interaction of the hydrophobic parts of the polymeric chains in the acrylic continuous bed and the hydrophobic cavity of the cyclodextrin derivatives. To decrease the hydrophobicity of the acrylic skeleton a fraction of IPA (less polar comonomer) was decreased in the comonomer mixture. Fig. 4 demonstrates the dependence of the elctroosmotic mobility on the pH value of the mobile phase for continuous beds with sulfated β-CD, where a ratio of hydrophobic IPA monomer to sulfated β-CD in the polymerisation mixture is changed. Small differences of electroosmotic mobility were observed for the continuous beds formed at different concentrations of sulfated β-CD 0.10 and 0.05 g/ml (Fig. 4, curves 1 and 2, respectively). The continuous bed formed at 0.10 g/ml of sulfated β-CD and twice lower (0.05 g/ml) concentration of IPA (Fig. 4, curve 3) exhibited moderate electroosmotic mobilities compared to the continuous bed represented by curve 1. The explana-

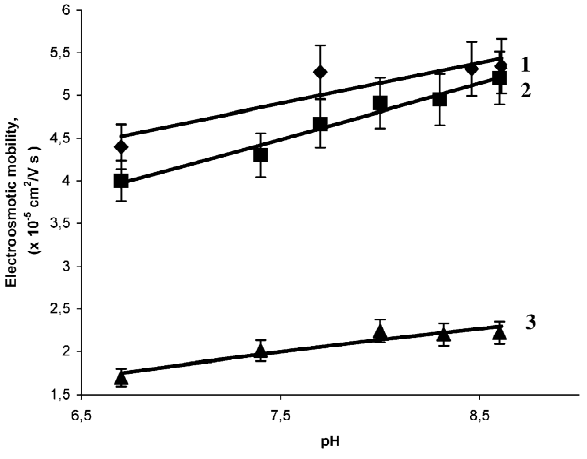

Fig. 4. Effect of pH on electroosmotic mobility in polyrotaxane continuous beds containing sulfated β-CD. Conditions: 50% aqueous MeOH, buffered with HOAc and TEMED; neutral marker: acetone, 5 kV; room temperature; conductivity adjusted to 165 μS/cm. I.D. = 50 μm. (1) Continuous bed formed at 0.1 g/ml of sulfated β-CD and 0.1 g/ml of IPA; (2) continuous bed formed at 0.05 g/ml of sulfated β-CD and 0.1 g/ml of IPA; (3) continuous bed formed at 0.1 g/ml of sulfated β-CD and 0.05 g/ml of IPA.

tion could be in the template-directed self-assembly process, when potential guest molecules form inclusion complexes already at 0.05 g/ml of sulfated β-CD (the host compound). This is an important observation, since two rotaxane formation mechanisms exist in terms of thermodynamics. One is when self-assembly of rotaxanes is spontaneous (entropy-controlled) another when it is template-directed (enthalpy-controlled) process. In the first case doubling of the macrocyclic compound concentration would result in a double yield of the threading, assuming, that the linear component is in excess. Generally, statistical threading can be applied to any linear polymer. This approach, however, is less favorable, when it is not driven by any intermolecular forces and results, therefore, in very low yields. The yield can be improved using the Le Chatelier's principle, when a large excess of either the cyclic or the linear component is employed [15].

It is well known, that cyclodextrins have apolar cavity and tend to interact with hydrophobic compounds/ligands forming inclusion complexes. Axial polymeric chains are formed from MA and IPA comonomers during the synthesis of continuous beds. Crosslinking of the complexes formed with PDA, which is present in the polymerisation mixture, prevents dethreading of cyclodextrins from the acrylic polymer chain and forms a solid three-dimensional structured polyrotaxane, consisting of cyclodextrin threaded on the skeleton of the acrylic polymer. The molecular modeling of the polyrotaxane structure with two β-CD rings threaded on the acrylic polymer network gave the a spatial image presented in Fig. 5. The molar ratios of the comonomers and crosslinker in the image corresponds to the standard procedure as given in the experimental part.

A template directed rotaxane formation is confirmed by the decreased electroosmotic mobility, when the continuous bed is formed at a 2-fold lower concentration of the nonpolar comonomer IPA, i.e. molar ratio of sulfated β-CD to IPA $m/n = 0.12$ instead of $m/n = 0.06$ (see the corresponding curves 1 and 3 in Fig. 4). The electroosmotic mobility, which was measured using acetone as an unretained marker, is approximately twice smaller through the pH range investigated. The electroosmotic mobility, which is dependent on the concentration of the charged macrocycles in the polyrotaxane-based continuous beds, indicates that polyrotaxane formation is favoured by hydrophobicity of the continuous bed skeleton. Less hydrophobic skeleton causes less number of macrocyclic macromolecules threaded and therefore lower concentration of the charged sulfate groups. The 2-fold decrease in the ratio between macrocycles and hydrophobic monomers ($m/n = 0.03$) did not change the electroosmotic mobility, as evident from the curves 1 and 2 in Fig. 4. This is not in agreement with the principle of statistical threading and rather indicates an enthalpy driven self-assembly (a complete threading or saturation of the linear polymer with macrocycles is reached at 0.05 g/ml of sulfated β-CD). A further increase of the concentration up to 0.10 g/ml does not, therefore, change the polyrotaxane composition. This is not unexpected, since it is known [15], that statistical threading occurs when identical or very closely related cyclic and linear compounds are used, for instance hydrocarbon macrocycle and hydrocarbon linear polymer. The structures obtained are referred to as homorotaxanes. Contrary, heterorotaxanes are formed from different cyclic and linear entities, mostly due to enthalpy driven self-assembly. The chain transfer is less likely here, since the EOF depends on the matrix hydrophobicity, which is directly related to the self-assembly process. It was no statistical EOF dependence on the charged cyclodextrin concentration in the polymerisation mixture. The chain transfer forming soluble polyrotaxanes was not observed by Gibson et al. [17] during the free-radical polymerization of the acrylic comonomers.

3.3. Electrochromatographic separations

Experiments with three homologues of alkylphenones or benzoic acid esters showed, that chromatographic separation with monolithic beds synthesized is in accordance with the reversed-phase mode (Fig. 6). Less polar homologues were retained more. Separation efficiency was around 100 000–140 000 theoretical plates/m for the retained compounds. Interestingly, a blank continuous bed formed without the sulfated β-CD additive gave no electroosmosis: acetone as neutral marker was not eluted within 4 h at the conditions identic to that shown in Fig. 6.

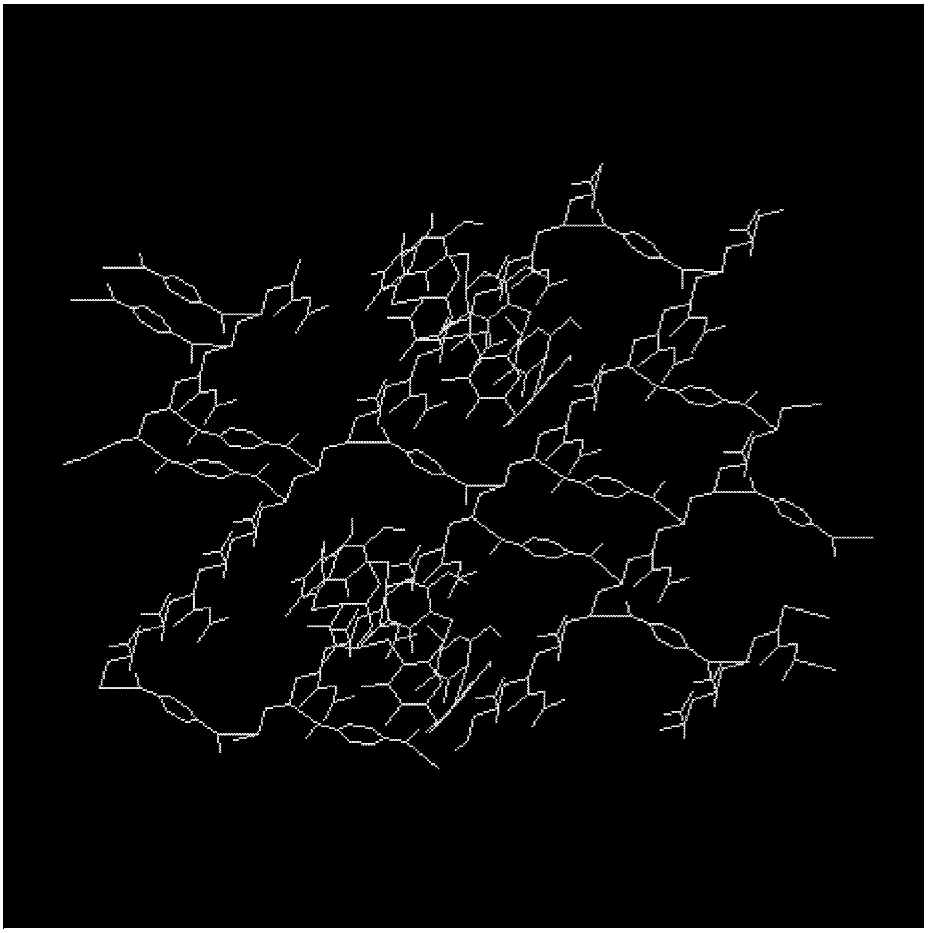

Fig. 5. Spatial structure of a polyrotaxane derivatized acrylic continuous bed. The molar ratio of the comonomers and crosslinker is given in the experimental section.

In order to investigate chiral recognition properties of the prepared capillary columns we used polyrotaxane-based continuous bed with neutral β-CD. Enantioseparation for a few compounds was obtained screening a range of chiral drugs under CEC conditions. Separation of racemic metoprolol is illustrated in Fig. 7. This is an interesting observation, since the macrocycle in the rotaxane structure is stranded by the axial unit, therefore the enantioseparation indicates that the outer surface and the hydrophilic rims of the cyclodextrin play a more important role in enantiorecognition than generally assumed. Moderate efficiency or tailing of the peaks was observed for the separated enantiomers, which can be explained by heterogeneity of the active centers in the polymeric network in the bed formed of different comonomers or by the varying degree of substitution in cyclodextrin derivatives. The accessibility of the chiral selectors may also differ in the bed. Recent results obtained separating enantiomers using pressure driven capillary chromatography confirmed the enantioselective properties of the polyrotaxane derivatized continuous beds.

3.4. Stability tests of the polyrotaxane derivatized continuous beds

In order to investigate the stability of the polyrotaxane-modified continuous beds the reproducibility of the electroosmotic flow was measured using

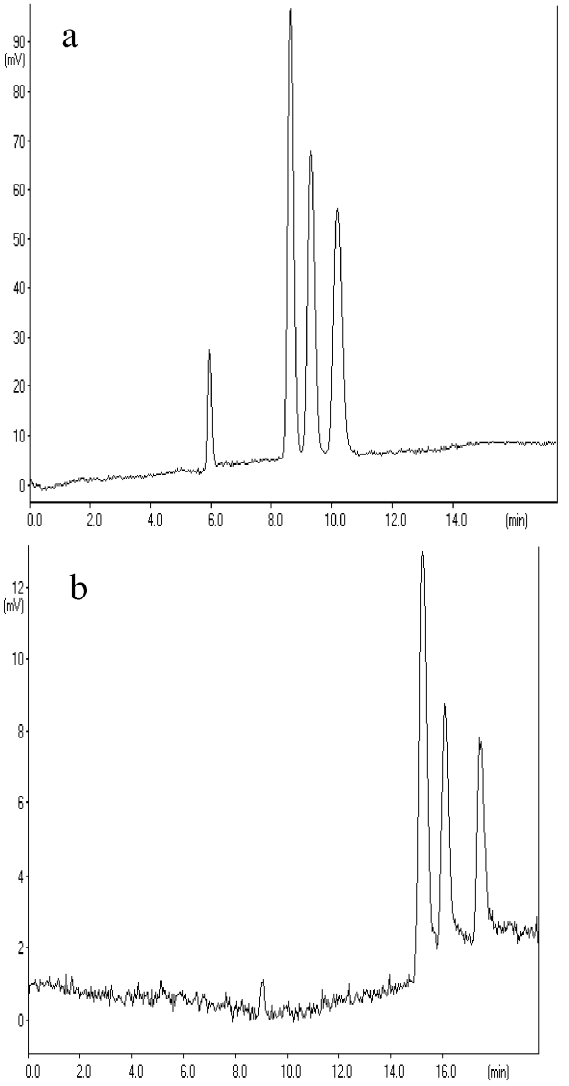

Fig. 6. Separation of alkylphenones (a) and benzoic acid esters (b) in CEC RP mode, using polyrotaxane derivatized continuous bed. Capillary: polyrotaxane continuous bed with 6-amino β-CD; mobile phase: MeOH–water (50:50, v/v), buffered with 0.05% TEMED and 0.1% HOAc (pH 5.1); voltage: 5 kV. Sample: (a) a mixture of acetone and alkylphenones (elution order: acetone, acetophenone, propiophenone and butyrophenone); (b) a mixture of benzoic acid esters (elution order: methyl-, propyl- and butyl-benzoic acid esters).

acetone as a neutral marker. No systematic change of the electroosmosis was observed in the course of several weeks (3-day systematic measurements for total number of 90 experiments resulted RSD=

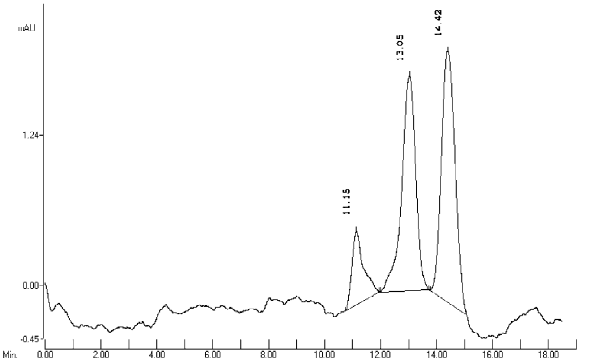

Fig. 7. Capillary:polyrotaxane continuous bed containing neutral β-CD and vinylsulfonic acid as ionic comonomer in polymerization mixture. Mobile phase: 50% MeCN in 0.05% TEA and HOAc, pH 4.5. Voltage: 5 kV. Curent: 3 μA. Sample: D,L-metoprolol, $t_0 = 11.15$ min.

0.69%), which confirms a covalent stability of the polyrotaxane formations in the continuous beds in contrast to physical entrapment, which usually causes bleeding [35] and continuous changes of the chromatographic properties of the stationary phase during the chromatographic separations. The beds were not degrading when dried out. After reconditioning the capillary columns were used in further CEC experiments. The stability of the sulfated β-CD containing polyrotaxane based continuous beds in extreme pH media was tested. No changes of the electroosmotic and chromatographic properties were observed within the pH range from pH 4 up to pH 9. Drop of the electroosmotic flow (more than four times) was observed for the beds pretreated in alkaline medium at pH 12.3 for 20 h (Fig. 8), which is not unexpected. It was demonstrated earlier by Hjertén et al., that continuous beds start to degrade at pH 12, when PDA as crosslinking agent is used [36]. This could be a reason for a partial release of the cyclodextrin macrocycles from the continuous bed hydrolysed under alkaline conditions.

4. Conclusions

In this study a multifunctional method for an one-step synthesis of different polyrotaxane-modified continuous beds has been demonstrated. Various stationary phases with desired electroosmotic and

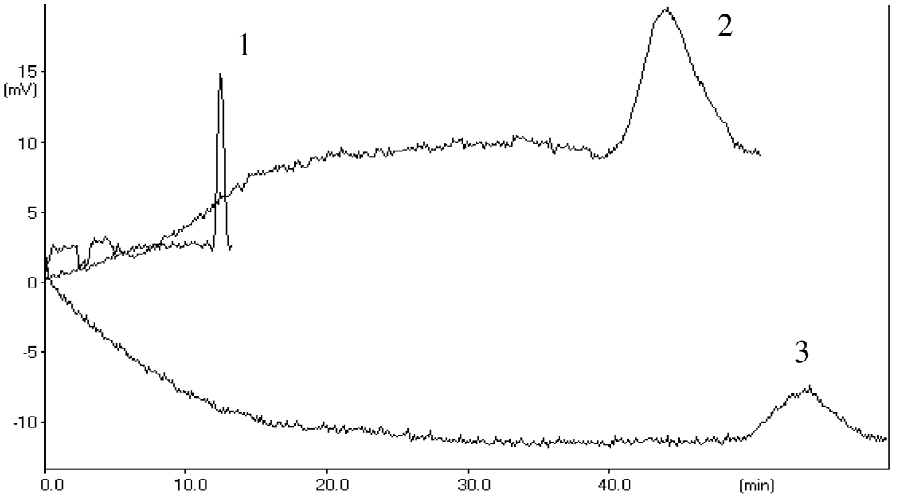

Fig. 8. Test of the polyrotaxane derivatized continuous bed stability at extreme pH values. Capillary: continuous bed with sulfated β-CD, effective length = 10.4 cm, total length = 14.5 cm, I.D. = 50 μm. Conditions: 50% MeOH buffered with HOAc and TEMED, pH 8.7, neutral marker: acetone, 5 kV, temperature 20 °C, the conductivity was adjusted to 165 μS/cm. (1) new column; (2, 3) column following washing with 0.1 *M* sodium hydroxide solution (pH 12.3), 20 h. Mobility decreased from $4.05 \cdot 10^{-5}$ cm^2/Vs to $0.93 \cdot 10^{-5}$ cm^2/Vs.

chromatographic properties were synthesized in this way, selecting the appropriate derivative of the macrocyclic compound in the polymerisation mixture. There is no need for additional reactions to form the rotaxanes, since they are formed during the synthesis of the crosslinked acrylic polymer matrix. The self-assembly of the polyrotaxane during the polymerisation of acrylic polymers in the presence of β-cylodextrin derivatives shows, that it is enthalpy controlled and template directed. Reversed-phase capillary electrochromatography was performed using polyrotaxane-based continuous beds. Although not primarily designed for this purpose, the polyrotaxane modified continuous beds have shown enantiorecognition properties. This study suggests, that polyrotaxane formation technique is a convenient and promising method to generate the desired properties in the crosslinked polymeric matrices.

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