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Short Communication

Voltage-assisted capillary LC of peptides using monolithic capillary columns prepared by ring-opening metathesis polymerization

We examined the use of monolithic capillary columns prepared *via* ring-opening metathesis polymerization (ROMP) for peptide separation in voltage-assisted capillary LC (voltage-assisted CLC). In order to demonstrate their potential for peptide separation, ROMP-derived monoliths with RP properties were prepared. The preparation procedure of monoliths was transferred from ROMP monoliths optimized for CLC. ROMP monoliths were synthesized within the confines of 200 μm id fused-silica capillaries with a length of 37 cm. After optimization of the chromatographic conditions, the separation performance was tested using a well-defined set of artificial peptides as well as two peptidic mixtures resulting from a tryptic digest of BSA as well as a collagenase digest of collagen. ROMP monoliths showed comparable performance to other monolithic separation media in voltage-assisted CLC published so far. Therefore, we conclude that by optimizing the composition of the ROMP monoliths as well as by using the controlled manner of their functionalization, ROMP monoliths bear a great potential in CLC and CEC.

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CEC [1] is a technique combining the high selectivity of HPLC and the high efficiency of CE. Since the first reports on CEC by Jorgenson and Lukacs [2] in 1981 as well as by Tsuda *et al.* [3] in 1982, CEC has become a powerful separation technique [4–6]. However, the speed of development of CEC has been hampered by practical problems and difficulties [7–9] mainly associated with (i) quantitative sample introduction without an injection valve [7]; (ii) the nontrivial preparation procedure of miniaturized packed columns and (iii) the creation of end-frits, which often limits the repeatability of separation. Furthermore, bubble formation at end-frits may result in ghost peaks and dry-out phenomena due to Joule heating. In order to overcome problems related to bubble formation, a pump can be coupled to the CEC system resulting in a hybrid technique known as pressurized CEC (pCEC) [5, 10–12]. In the literature, two terms for this tech-

nique are used, namely pCEC (pressure-assisted) and voltage-assisted CLC. The differentiation between these two terms is based on the dominant contribution to the mobile phase flow, *e.g.*, EOF in the case of pCEC and pressure in the case of voltage-assisted CLC [13].

A number of reviews on CEC have been published recently [13, 14], clearly demonstrating its potential for peptide analysis. Herein, the increase in the number of applications using monolithic separation media instead of particle packed columns is striking. The main reason for this trend is due to the technical difficulties often associated with the preparation of packed capillary columns; *e.g.*, sophisticated packing procedures and the manufacturing of end-frits. Both steps certainly require experimental skills and experience to produce particle packed capillary columns in a reproducible way. In contrast, the ease of preparation of monolithic stationary phases is one of their great advantages over packed capillary columns. However, it is worth mentioning that for CEC and pCEC, charged separation media are needed to generate the EOF.

Several reviews are available, focusing on monolithic stationary phases, addressing both monolith characteristics and their application in CEC [13, 15–18]. Herein, different types of monoliths whether silica- or polymer-based, with diverse surface functionalities are discussed. With respect to the application of hydrophobic polymer-based monoliths,

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Abbreviations: CLC, capillary LC; ROMP, ring-opening metathesis polymerization

two contributions are of particular interest. Wu *et al.* [19] used a hydrophobic methacrylate-based monolithic column without any EOF for the separation of peptides. The ionic analytes were separated on the basis of their differences in electrophoretic mobility and hydrophobic interaction with the stationary phase. Szucs and Freitag [20] demonstrated the usage of neutral poly(glycidyl methacrylate-*co*-ethylene dimethacrylate)-based monoliths for the analysis of peptides using voltage-assisted capillary LC (CLC) and compared this method to the separation performance of CEC- and nano-HPLC-based systems. In their work, the potential of hydrophobic polymeric monoliths for peptide separation in voltage-assisted CLC has been clearly demonstrated.

The overall aim of this work was to elaborate the separation performance of hydrophobic ring-opening metathesis polymerization (ROMP)-derived monoliths as stationary phases for voltage-assisted CLC, in particular for the separation of peptides. ROMP-derived monoliths were first published in 2000 by Sinner and Buchmeiser [21, 22] and have since then predominantly been used for HPLC, nano-HPLC, SPE [23], and also for heterogeneous catalysis [24]. During the last years, the focus in separation science was on the optimization of ROMP-derived monolithic capillary columns for peptide separation by nano-HPLC [25]. Based on these results we herein report for the first time the preparation of RP ROMP-derived monoliths for the application in voltage-assisted CLC.

Fused-silica capillaries (nondeactivated, 365 μm od, 200 μm id) were purchased from J & W (Agilent, Palo Alto, CA, USA). Capillaries were cut into appropriate pieces; one end was equipped with a luer-lock adapter and tightly connected to the capillary *via* a PEEK-sleeve. To ensure anchoring of the monolith to the inner capillary wall, capillaries were pretreated as described previously [26–28]. Synthesis of ROMP monoliths was accomplished according to a previously published protocol [26] using [2.2.1]bicyclohept-2-ene, 1,4,4a,5,8,8a-hexahydro-1,4,5,8-*exo,endo*-dimethanonaphthalene, toluene, 2-propanol (10:10:10:70% m/m), and $\text{RuCl}_2(\text{PCy}_3)_2(\text{CHPh})$ (where Ph is phenyl, and Cy is cyclohexyl) (0.5% m/m). End capping was performed using vinyl ethyl ether and by subsequent washing with ACN.

Experiments were performed on a Beckman P/ACE system 5500 (Beckman, Fullerton, CA, USA). Detection was done by UV absorbance recorded at 214 nm. The length of the monolith was 30 cm (37 cm entire length of the capillary). Prior to analysis, the monolithic capillary was washed overnight with the mobile phase using a syringe pump (KD Scientific Inc., Holliston, MA USA) with a manual capillary rinsing tool (Supelco 55042, Bellefonte, PA, USA) at the flow rate of 30 $\mu\text{L}/\text{h}$. Samples were injected using the electrokinetic method (5 or 10 kV). Separations were performed at 20°C applying a voltage of 10 kV and a pressure of 138 kPa to the inlet end of the capillary. A citrate–phosphate buffer (25 mmol/L, pH 3.5, 20% ACN) was used. Three types of peptide mixtures were used to evaluate the separation performance of ROMP monoliths: (i) model mixture of nine synthetic oligopeptides, (ii) a tryptic digest of BSA (after pre-

treatment with dithithreitol and iodoacetic acid) [29] and (iii) a collagenase digest of the collagen type I [30].

The preparation of capillary monoliths for CEC and voltage-assisted CLC is more challenging than for capillary HPLC. The formation of a well-defined end of the monolith inside the capillary in order to generate the detection window is somewhat tricky. Therefore, the preparation of capillary ROMP monoliths for voltage-assisted CLC was realized by applying low pressure at one end of the capillary till the polymerization mixture reached the detection window. After the formation of the monolith, which takes place within 30 min, end capping of the initiator and extensive washing are needed to ensure the complete removal of the initiator and any unreacted monomer.

The RP ROMP monoliths used for our experiments did not bear charges and are therefore comparable to the neutral “C1”-type column described by Szucs and Freitag [20]. Thus, in order to evaluate the applicability of ROMP monoliths for voltage-assisted CLC, we used an approach similar to the published method. In the first step, a well-defined peptide standard, consisting of nine peptides (ranging from tri- to hexapeptides, M_r within the range of 240–1000) was used to show the potential of RP ROMP monoliths as a separation media in voltage-assisted CLC. Separation conditions were optimized in terms of buffer concentration (10–50 mmol/L), pH (3–9) as well as type, composition, and concentration of the organic solvents used as the mobile phase (methanol, 2-propanol, ACN). All of the parameters mentioned above had a significant influence on the separation performance. The influence of the pressure and voltage applied is shown in Fig. 1. It is evident that both are key parameters and their increase accelerates the separation of peptides. The EOF was determined by using DMSO as a marker. Migration time of this marker was 57 and 43.5 min, using 5 and 10 kV, respectively. Elution of peptides from the ROMP monolith was not possible by pressure alone (138 kPa), unless it was conjuncted with the EOF. Therefore, we assume that a low EOF was presumably generated by the free silanol groups of the short outlet part of the capillary. These findings are in accordance with the published results obtained with hydrophobic monoliths [19, 20] and are underlining that voltage-assisted CLC is the dominant separation technique in the case of RP ROMP monoliths.

By optimization of the chromatographic parameters (25 mmol/L citrate–phosphate buffer at pH 3.5 with 20% v/v of ACN applying a voltage of 10 kV and a pressure of 138 kPa, at 20°C), ROMP monoliths revealed only moderate separation efficiencies for selected peptides of up to 83 000 plates/m. However, a superior separation performance compared to peptide separation by HPLC was achieved.

In the second step, we transferred the optimized separation conditions to the separation of peptide mixtures resulting from enzymatic digests of proteins. The separation performances of RP ROMP monoliths for the tryptic digest of albumin and the collagenase digest of collagen type I are

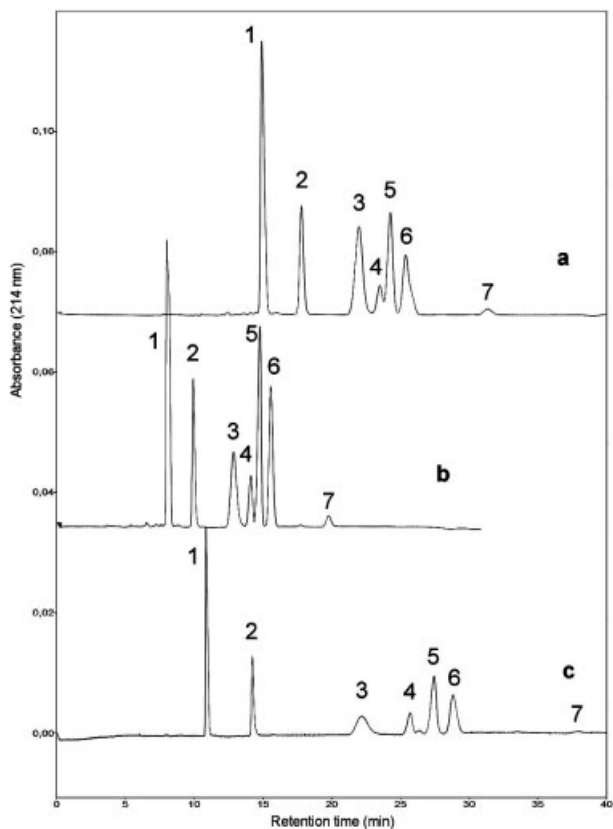


Figure 1. Influence of the voltage and pressure applied onto the separation of a well-defined mixture of synthetic oligopeptides. Identification: 1-Gly-Gly-His, 2-Arg-Pro-Pro-Gly-Phe, 3-Ala-Pro-Gly, 4-Gly-Gly-Gly-Gly, 5-Phe-Gly-Gly and Ala-Ala-Ala-Ala-Ala, 6-Gly-Phe-Phe and Phe-Gly-Phe-Gly, 7-Tyr-Tyr-Tyr-Tyr-Tyr. Sample injection: (a) electrokinetic method (5 kV); voltage: 5 kV; pressure: 138 kPa applied to the inlet end of the ROMP capillary, (b) electrokinetic method (10 kV); voltage: 10 kV; pressure: 138 kPa applied to the inlet end of the ROMP capillary, (c) electrokinetic method (5 kV); voltage: 10 kV; pressure: 3.45 kPa applied to the inlet end of the ROMP capillary.

shown in Fig. 2. As can be seen in the chromatograms, a good separation performance was obtained in both cases. In the case of BSA, 35 peaks (of 72 theoretically expected peptidic fragments) were detected, in the case of collagen type I more than 50 peaks (of 73 theoretically expected peptidic fragments) are present in the chromatogram.

According to these preliminary results and in view of the ease of preparation, low cost, and the reproducibility of the synthesis of ROMP monoliths, we conclude that voltage-assisted CLC using RP ROMP-derived monolithic capillaries is a promising method for the analysis of complex peptide mixtures. Due to these promising first results, ROMP monolith optimization for peptide and protein separation is currently under investigation. Furthermore, ongoing research projects will focus on the preparation of ROMP monoliths bearing different functional groups and on their applicability in CEC separations of biomolecules.

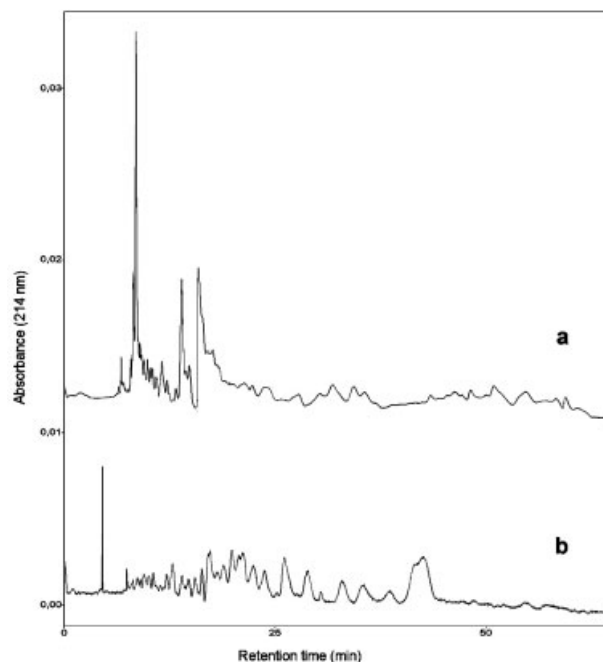


Figure 2. Separation of the enzyme digests of proteins. (a) Collagenase digest of collagen, (b) tryptic digest of BSA; chromatographic conditions: ROMP monolith: 30 cm, 200 μ m id, mobile phase: 25 mmol/L of citrate-phosphate buffer pH 3.5 containing 20% v/v ACN; temperature: 20°C; UV: 214 nm; sample injection: (a) electrokinetic method (5 kV); voltage: 10 kV; pressure: 138 kPa applied to the inlet end of the ROMP capillary; (b) electrokinetic method (10 kV); voltage: 10 kV; pressure: 138 kPa applied to the inlet end of the ROMP capillary.

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