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Journal of Chromatography A, 1090 (2005) 81–89
JOURNAL OF CHROMATOGRAPHY A
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Evaluation of ring-opening metathesis polymerization (ROMP)-derived monolithic capillary high performance liquid chromatography columns

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Received 24 February 2005; received in revised form 23 June 2005; accepted 30 June 2005
Available online 2 August 2005

Abstract

Novel monolithic capillary HPLC columns were prepared via ring opening metathesis polymerization (ROMP) within the confines of fused silica columns with 200 μm i.d. using norborn-2-ene (NBE), 1,4,4a,5,8,8a-hexahydro-1,4,5,8, *exo, endo*-dimethanonaphthalene (DMN-H6) as monomers, 2-propanol and toluene as porogens and $RuCl_2(PCy_3)_2(CHPh)$ as initiator. Using the monolithic capillary HPLC columns, different sets of analytes (i.e. standard systems) were used for the evaluation of the monolithic columns: (i) a protein standard consisting of six proteins in the range of 5000–66 000 g/mol, (ii) an insulin–albumin standard, and (iii) a peptide standard obtained from a tryptic digest of cytochrome C. With these three different standard systems the reproducibility of synthesis in terms of separation performance proved to be 1–2% relative standard deviation in t_R. Variation of polymerization parameters had a significant influence on the monolithic morphology and therefore separation efficiency and back pressure. The maximum analytical loading capacity of ROMP-derived monolithic capillary columns for albumin was found to be 30–125 ng, depending on the monomer content. Long-term stability studies showed no alteration in separation performance.

Keywords: Stationary phases; LC; Monolithic columns; Capillary columns; Metathesis polymerization; Proteins; Peptides

1. Introduction

Monolithic separation media have become powerful tools in separation science. Both silica and polymer-based systems are available [1–11], allowing the separation of both low and high molecular weight analytes. In comparison to conventional packed columns, particular advantages of monolithic separation media result from their unique structure. As they consist of one single piece of highly porous material, an enhanced mass transport is achieved [12–16]. In addition, the lower backpressure enables fast chromatographic separations allowing higher flow rates without loss in efficiency. Furthermore, the simple preparation process is especially favorable for miniaturization e.g. the preparation of capillary columns. In order to contribute to both miniaturization and fast analysis, either micro-(capillary) or disc based monolithic systems are currently under investigation [17–28]. While the former is usually used in combination with mass spectrometry, the latter combines comparably high capacity with high speed and good resolution [29–34]. Particularly quantification by mass spectroscopic techniques

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doi:10.1016/j.chroma.2005.06.098

and sequencing with the aid of standard data files are standard techniques in proteomics, however, these are dramatically facilitated in case highly efficient separation devices can be applied [35–38]. So far, our contribution to the field was the introduction of monolithic, organic stationary phases prepared by a transition-metal catalyzed polymerization process, i.e. ring-opening metathesis polymerization (ROMP) [39,40], allowing the manufacture of monolithic supports in the range of 200 μm–5 cm in terms of inner diameter (i.d.) with basically no restrictions in length [41–47]. In depth investigations on the influence of polymerization parameters on separation behavior and efficiency have already been carried out for monolithic phases with an i.d. ≥3 mm, however, this remained to be performed for monolithic columns with i.d.'s <3 mm, particularly for those in the micrometer range. In view of ongoing projects involving the coupling of our monolithic phases to mass spectrometry, it is now that we can report on these investigations performed with capillary HPLC columns with an i.d. of 200 μm. For chromatographic evaluation, three different standard systems were chosen: (i) a protein standard consisting of six proteins in the range of 5000–66 000 g/mol (i.e. ribonuclease A, insulin, albumin, lysozyme, myoglobin, ß-lactoglobulin), (ii) an insulin–albumin standard (bovine insulin, porcine insulin, human insulin, human albumin), and (iii) a peptide standard obtained from a tryptic digest of cytochrome C. With these three different standard systems the reproducibility of synthesis in terms of separation performance, the influence of changes in polymerization parameters on separation efficiency and back pressure were investigated. In addition, loading and long-term stability studies were conducted.

2. Experimental

2.1. Chemicals, reagents and samples

[2.2.1]Bicyclohept-2-en-5-ylmethyldichlorosilane (95%) was purchased from Gelest Inc. (Morrisville, PA, USA). [2.2.1]Bicyclohept-2-ene (norborn-2-ene, NBE) (97%), NaOH (99%), acetone (99.8%), toluene secco solv., 2-propanol secco solv., triphenylphosphine (PPh_3) (98.5%) and acetonitrile LiChrosolv were purchased from Merck (Darmstadt, Germany). Pyridine (99%) was purchased from Pierce (Rockford, IL, USA), methanol (99.7%) from Riedel-de Haën (Seelze, Germany). Trypsin was purchased from Promega (Madison, WI, USA). The crosslinker 1,4,4a,5,8,8a-hexahydro-1,4,5,8, *exo*, *endo*-dimethanonaphthalene (DMN-H6) was prepared from freshly cracked dicyclopentadiene and pure norbornadiene (both Fluka, Buchs, Switzerland) according to the literature [48]. Ethyl vinyl ether (99%), $RuCl_2(PCy_3)_2(CHPh)$ (Cy = cyclohexyl) (97%), water (HPLC-grade), trifluoroacetic acid (99.5%), ribonuclease A (from bovine pancreas), lysozyme (from chicken egg white), albumin (from bovine serum), albumin (from human serum), myoglobin (from horse skeletal muscle), ß-lactoglobulin (from bovine milk), insulin (from bovine pancreas), insulin (from porcine pancreas), insulin (human recombinant expressed in yeast), cytochrome C (from bovine milk), urea (99.7%), ammonium bicarbonate (99%) and the peptide separation buffer (PSB) were purchased from Sigma–Aldrich (Vienna, Austria).

For the preparation of the tryptic digest of cytochrome C, urea (12.0 g, 120 mmol) and NH_4HCO_3 (0.791 g, 10 mmol) were dissolved in 100 ml of H_2O (solution 1). Trypsin (20 μg, 0.833 nmol) was treated with 50 mM acetate buffer and subjected to shaking at 37 °C for 30 min. Cytochrome C was denatured in 0.5 ml of solution 1. The solution of trypsin was treated with 0.5 ml of solution 1 and the mixture was combined with the solution of cytochrome C. The final mixture was subject to shaking at 37 °C for 24 h. Trifluoroacetic acid (1 ml, 0.1%) was added to terminate the digest. The standard solution was further diluted with mobile phase. All other standards were prepared by dissolving them in peptide separation buffer and further dilution with mobile phase. All standards were stored at −80 °C.

2.2. High-performance liquid chromatography

Fused silica capillaries (non deactivated, 365 μm o.d., 200 μm i.d.), were purchased from J & W (Agilent, Palo Alto, CA, USA). The micro-splitter valve as well as the PEEK sleeves and ferrules were from Upchurch Scientific (Oak Harbor, WA, USA). The luer-lock-adapter was purchased from VICI Jour (Onsala, Sweden). The chromatographic system consisted of an autosampler GINA 500, a HPLC pump P 580A LPG, a column oven STH 585 (all Dionex, Sunnyvale, CA, USA) and an Ultimate UV-detector equipped with a nano-flow cell (LC Packings, Amsterdam, The Netherlands). Data acquisition was accomplished using a Chromeleon software package (Version 6.40). The primary flow rate was split using a micro-splitter valve and a restriction capillary. The resulting flow rate through the column was measured after UV-detector using a Hamilton syringe (Hamilton, Bonaduz, Switzerland). Electron microscopy was carried out at the Center for Electron Microscopy, ZFE, Graz, Austria.

2.3. Synthesis of ROMP-based monolithic capillary columns

Capillaries were cut into appropriate pieces, one end was equipped with a luer-lock adapter, tightly connected to the capillary via a PEEK-sleeve. Capillaries were flushed with water, then etched with NaOH (1 M) for 4 min at room temperature, again flushed with water and dried at 60 °C applying a continuous stream of N_2 for 1 h. Surface modification was accomplished applying a mixture of pyridine/toluene/[2.2.1]bicyclohept-2-en-5-ylmethyldichlorosilane (molar ratio 3.5:1.7:1) at 60 °C over night. Finally, silanized capillaries were consecutively washed with acetone, water and methanol and dried at 40 °C under a stream of N_2 for 1 h. Capillaries prepared by this

method may be stored over months. Synthesis of monoliths were accomplished, according to a previously published protocol using NBE, DMN-H6, toluene, 2-propanol and $RuCl_2(PCy_3)_2(CHPh)$ [44]. For the actual composition of the different monoliths please refer to Table 2. The length of the monolithic capillary columns was 14 cm for all investigations (except for backpressure measurements, here the length was 13 cm).

3. Results and discussion

In comparison to conventionally used analytical HPLC systems, capillary-HPLC (cap-HPLC) offers the advantage of higher sensitivity. In terms of monoliths, capillary supports show several advantages compared to monolithic supports in analytical dimensions. On one hand capillary monoliths offer increased mechanical stability due to the large surface to volume ratio. In addition, they usually possess more homogeneous fillings, which in due consequence result in an increase in separation efficiency [49]. In principle, recipes in terms of polymer, respectively polymer precursor composition identical to those used in the manufacture of large bore columns may be used for the synthesis of small scale systems, being typically in the range of 50–200 μm.

In order to make a profound statement on the influence of changes of polymerization parameters on separation performance, both run-to-run reproducibility (i.e. reproducibility of the cap-HPLC system) and column-to-column reproducibility (i.e. reproducibility of monolith synthesis) of ROMP-derived capillary columns were checked. Identification of the influences of changes of polymerization parameters on separation performance was accomplished by chromatographic techniques, changes in morphology were investigated by electron microscopy. Final investigations were dedicated to loading capacity and long-term stability.

3.1. Run-to-run precision

We used a self-made cap-HPLC system as shown in Fig. 1. The setup consisted of a conventional HPLC system with an additional micro splitting valve. Therefore, to specify imprecision resulting from the self-made cap-HPLC system, run-to-run precision for monolith **1–5** was checked by three-fold, consecutive injection of all three standard systems. Table 1 summarizes the data for run-to-run precision of all three standard systems.

Table 1
Summary of values for run-to-run reproducibility

Standard	t_R [min]	$w_{1/2}$ [min]	S	R
Protein	0.2–1.4	0–19.7	0–31	0.3–33
Insulin/albumin	0.2–6.3	1.4–6[a]	0.2–7.1[a]	
Tryptic digest of cytochrom C	0.1–2.7	0–25.3	0.8–24.3	0.7–22.9

Relative standard deviation [%] of chromatographic parameters derived from monoliths **1**–**5**, values for tryptic digest were derived from monoliths **1**–**4**. Chromatographic conditions: mobile phase: (A) 95% water, 5% acetonitrile, 0.05% TFA, (B) 20% water, 80% acetonitrile, 0.04% TFA; flow 1 μl/min; 25 °C; detection, UV 190 nm. Gradient for protein separation: 0–30 min 0–60% (B); 60–90% (B) within 5 min. Gradient for insulin separation: 0–30 min 29–33% (B); 33–90% (B) within 5 min. Gradient for peptide separation: 0–30 min 0–50% (B); 50–90% (B) within 5 min.

[a] Values obtained for albumin only.

The variability, expressed in relative standard deviation, in retention time t_R for the protein standard consisting of ribonuclease A, human insulin, lysozyme, bovine albumin, myoglobin and ß-lactoglobulin was exceptionally low for a self-made cap-HPLC system (0.2–1.4%). Some larger deviations were observed in both peak width ($w_{1/2}$) and symmetry (S), a fact related to the occurrence of ghost peaks. ß-Lactoglobulin was an exception in that it was separated into two peaks, representing different variants, resulting in a lower reproducibility in $w_{1/2}$ and S (Fig. 2). The insulin/albumin standard consisted of three insulin homologues (bovine insulin, human insulin, porcine insulin) and human albumin. Run-to-run precision was again satisfactory, however, the variability in t_R of the homologous insulins was slightly higher compared to the protein standard. However, the variability in t_R for albumin was comparable to other standards (RSD < 2%). This increase in t_R for insulin is believed to result from the gradient mixer due to the rather flat solvent gradient (solvent B: 29–33% in 30 min, to 90% within 5 min), which was chosen in order to avoid continuous and time-consuming changes

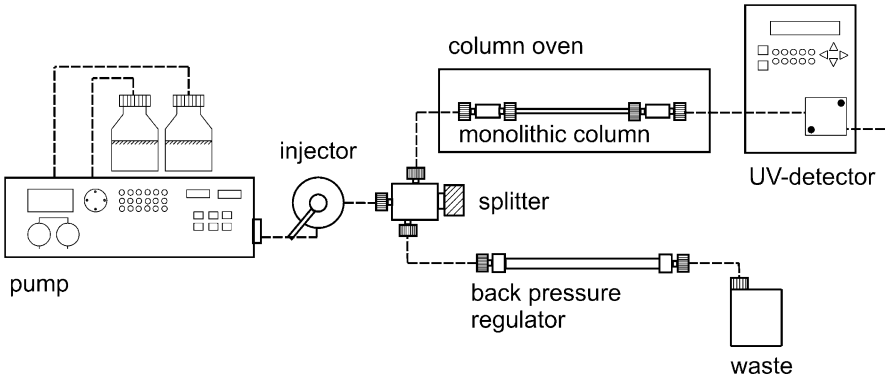


Fig. 1. Experimental setup for self-made cap-HPLC.

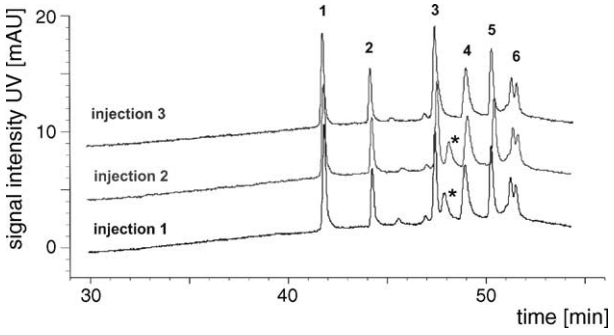


Fig. 2. Run-to-run precision of the protein standard on monolith **3**, threefold injection. (1) Ribonuclease A, (2) insulin, (3) lysozyme, (4) albumin, (5) myoglobin and (6) ß-lactoglobulin, *ghost peak, 2–8 ng of each protein. Chromatographic conditions: mobile phase: (A) 95% water, 5% acetonitrile, 0.05% TFA, (B) 20% water, 80% acetonitrile, 0.04% TFA; gradient: 0–30 min 0–60% (B); 60–90% (B) within 5 min; flow 1 μl/min; 25 °C; detection, UV 190 nm.

of the solvent. Since the homologous insulins could not sufficiently be separated, the values for $w_{1/2}$, S, and R could not be determined. For the separation of the tryptic digest of cytochrome C five peaks were chosen for run-to-run and column-to-column determination. Regarding those five peaks a low variability in t_R of 0.1 – 2.7% was observed. The relative standard deviations of all other parameters were significantly higher and varied from peak to peak. However, variability's of retention times for all standard systems on monolith 1–5 were well within the values characteristic for cap-HPLC systems [23].

3.2. Reproducibility of synthesis

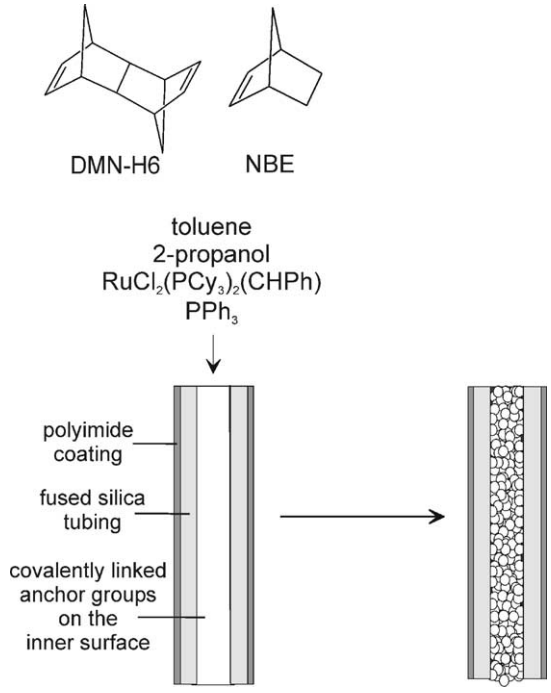
Reproducibility of synthesis is an important issue in case monolithic columns are used in routine analysis, since high reproducibility guaranties constant separation characteristics. Having quantified the precision of the self-made cap-HPLC, a comparison between different monoliths prepared by the same recipe was possible. This was anticipated to reveal the influence of the manual (i.e. non-automated) manufacturing process on separation performance. For this purpose, five monoliths (**1**–**5**) of identical composition (Table 2) were synthesized (Scheme 1) and subjected to chromatographic testing. Particular attention was given to column-to-column reproducibility and back pressure.

All data obtained on monoliths **1**–**5** were pooled. When interpreting reproducibility of synthesis data one has to keep in mind that the data obtained is the sum of run-to-run precision and column-to-column reproducibility. For all standard systems, the relative standard deviation for t_R increased only slightly (for proteins in average 0.9%, for peptides in average 1.4%, for insulin in average 7.3%, for albumin 0.3%). Thus, except for insulin, the column-to-column precision of retention times for all investigated analytes revealed the excellent reproducibility of synthesis. For all other parameters ($w_{1/2}$, S, R) the increase was more pronounced, however, a satisfactory reproducibility was observed (Table 3).

Table 2
Composition of monoliths **1**–**20**[a]

Monolith no.	NBE [%]	DMN-H6 [%]	2-PrOH [%]	Toluene [%]	Ph_3P added [ppm][b]
1–5	25	25	40	10	40
6	25	25	40	10	20
7	25	25	40	10	40
8	25	25	40	10	80
9	25	25	40	10	160
10	10	10	70	10	40
11	15	15	60	10	40
12	20	20	50	10	40
13	25	25	40	10	40
14	30	30	30	10	40
15–17	10	10	70	10	40
18–20	25	25	40	10	40

[a] [Initiator] = 0.5 wt.% throughout.
[b] (w/w).



Scheme 1. Synthesis of monolithic capillary HPLC columns via ROMP.

Table 3
Summary of values for column-to-column reproducibility

Standard	t_R [min]	$w_{1/2}$ [min]	S	R
Protein	1.2–2	13.3–30.4	7.3–31.7	15.6–27.5
Insulin/albumin	0.8–9.7	11.2–15.5	9.8	
Tryptic digest of cytochrom C	1.8–3.4	5.2–26	8.6–33.1	7.7–49.4

Relative standard deviation [%] of chromatographic parameters derived from monoliths **1**–**5**, values for tryptic digest were derived from monoliths **1**–**4**. Chromatographic conditions: mobile phase: (A) 95% water, 5% acetonitrile, 0.05% TFA, (B) 20% water, 80% acetonitrile, 0.04% TFA; flow 1 μl/min; 25 °C; detection, UV 190 nm. Gradient for protein separation: 0–30 min 0–60% (B); 60–90% (B) within 5 min. Gradient for insulin separation: 0–30 min 29–33% (B); 33–90% (B) within 5 min. Gradient for peptide separation: 0–30 min 0–50% (B); 50–90% (B) within 5 min.

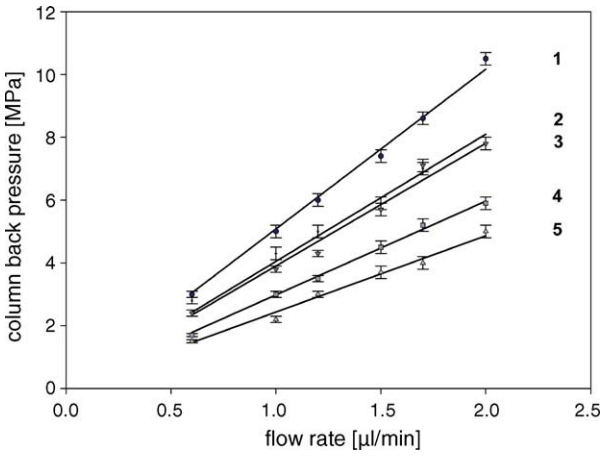


Fig. 3. Effective back pressure vs. flow rate for monoliths **1**–**5**. Mobile phase: water. For their synthesis, refer to Table 2.

Comparing the reproducibility of synthesis of capillary monoliths with ROMP-monoliths in the analytical format [44], capillary monoliths show an enhanced column-to-column reproducibility. In comparison to other capillary monolithic columns a significantly enhanced column-to-column reproducibility for ROMP-based capillary monoliths could be observed. Oberacher et al. reported an average standard deviation of 9.5% for retention times of $(dT)_{12–18}$ among 10 batches of monolithic poly(styrene-co-divinylbenzene) capillary columns [23].

3.2.1. Back pressure

The back pressure of a monolithic column is a crucial parameter. As a cumulative parameter, it directly reflects the morphology of the stationary phase in terms of pore volume and pore size distribution. The latter are a direct consequence of the monolith composition in terms of monomers, solvents and catalysts as well as of polymerization kinetics. Finally, plotting flow rate versus back pressure provides information about the compressibility of a stationary phase. Fig. 3 shows this graph for monoliths **1**–**5**.

A strictly linear behavior, indicative for the non-compressibility of the monoliths, was observed in all cases.

3.3. Influence of polymerization parameters on the separation of biomolecules

As already outlined in previous disclosures [43,44], the microstructure of monolithic supports via ROMP is highly dependant on the ratio respectively the amount of monomer and crosslinker, the ratio and amount of porogens, on initiator concentration and polymerization temperature. Any change in one of these parameters has a significant influence on the resulting morphology.

Taking advantage of information retrieved from previous studies [42,43], two important parameters have been investigated:

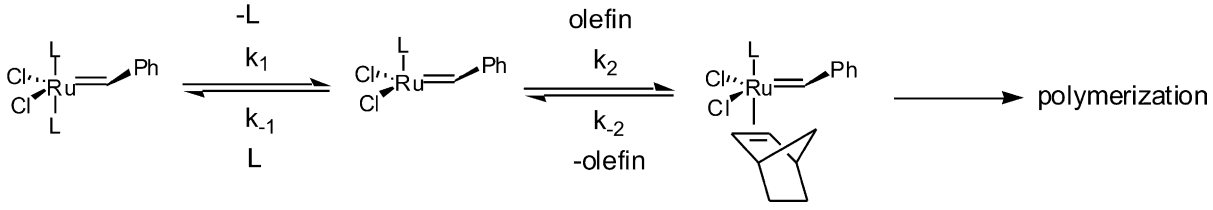
1. Change of polymerization kinetics by adding excess of phosphine (PPh_3).
2. Change of the monomer plus crosslinker content.

The influence of changes in these parameters on the microstructure of the monolithic columns was investigated by chromatography applying all three standard systems (i.e. protein standard, insulin/albumin standard, and a tryptic digest of cytochrome C). In addition, changes in the effective back pressure were measured. Finally, the monolithic supports were characterized by means of electron microscopy.

3.3.1. Changes in polymerization kinetics

The polymerization mechanism of $RuCl_2(PCy_3)_2(CHPh)$, the initiator used in these studies, is well understood. It proceeds via a dissociative mechanism, where one phosphine needs to dissociate from the parent complex to generate a free coordination site at the catalyst, which is in due consequence used for the coordination of a monomer (Scheme 2).

All these processes are reversible throughout with $k_1 = 0.38\,s^{-1}$, $k_{-1} = 0.62\,s^{-1}$ and $k_B = 0.16\,s^{-1}$ (phosphine exchange rate, $T = 37\,^{\circ}C$) [50,51]. With these ratios of rate constants given it is evident that the entire system is highly dependant on phosphine concentration. Any increase in phosphine concentration therefore leads to a significant reduction in the observed overall rate of polymerization k_p. This does not only allow the reaction heat to dissipate more homogeneously, it also influences morphology in that it provides more time for the monomer, the crosslinker and toluene to diffuse into the growing microglobules. As a consequence, the particle diameter increases and the volume fraction of pores (ε_p), the pore volume (V_P) and the specific surface area (σ_s) decrease. On the contrary, the volume fraction of interparticle void volume (ε_z) increases [44] (for an illustration of the discussed parameters see Fig. 4). This translates into a reduced back pressure and has a strong influence on the separation capability of a monolith. At low phosphine con-



Scheme 2. Mechanism of ROMP using $RuCl_2(PCy_3)_2(CHPh)$, L = ligand (PCy_3), Ph = Phenyl, Cy = Cyclohexyl.

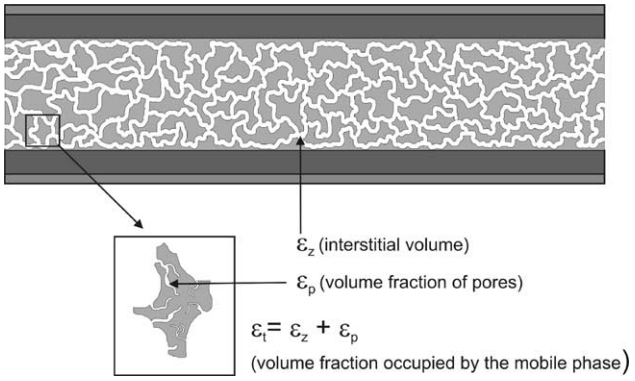


Fig. 4. Illustration of the physical meanings of the interstital volume (ε_z), the volume fraction of pores (ε_p) and the volume fraction occupied by the mobile phase (ε_t).

centration, the separation capabilities of monoliths **6** and **7** (20 and 40 ppm (w/w) PPh_3) are basically identical, which correlates with the apparently identical structures observed by electron microscopy (Fig. 5).

However, an increase in phosphine content from 40 to 160 ppm resulted in an increase in microglobule diameter from 1 to 2.6 μm and a change in morphology from "agglomerated" to "spherical" (Fig. 5, monoliths **8** and **9**). In due consequence and in accordance with previous findings [42–44,47], this change in morphology is accompanied by a dramatic loss in t_R and separation efficiency, apparently a direct consequence of the loss of porosity and specific

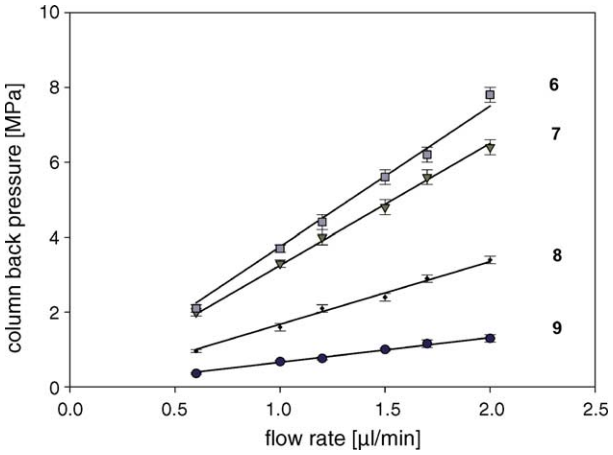


Fig. 6. Effective back pressure vs. flow rate for monoliths **6–9**. Mobile phase: water. For their synthesis, refer to Table 2.

pore volume. Particularly compounds with low molecular weight (<10 000) are affected, which is easy to understand since retention of these compounds is strongly affected by changes in micro- and meso-porosity. As expected, this is also reflected by a reduction in back pressure (Fig. 6).

3.3.2. Influence of monomer content on separation efficiency

Among others, the monomer (i.e. NBE plus crosslinker) content is one of the important parameters that determine

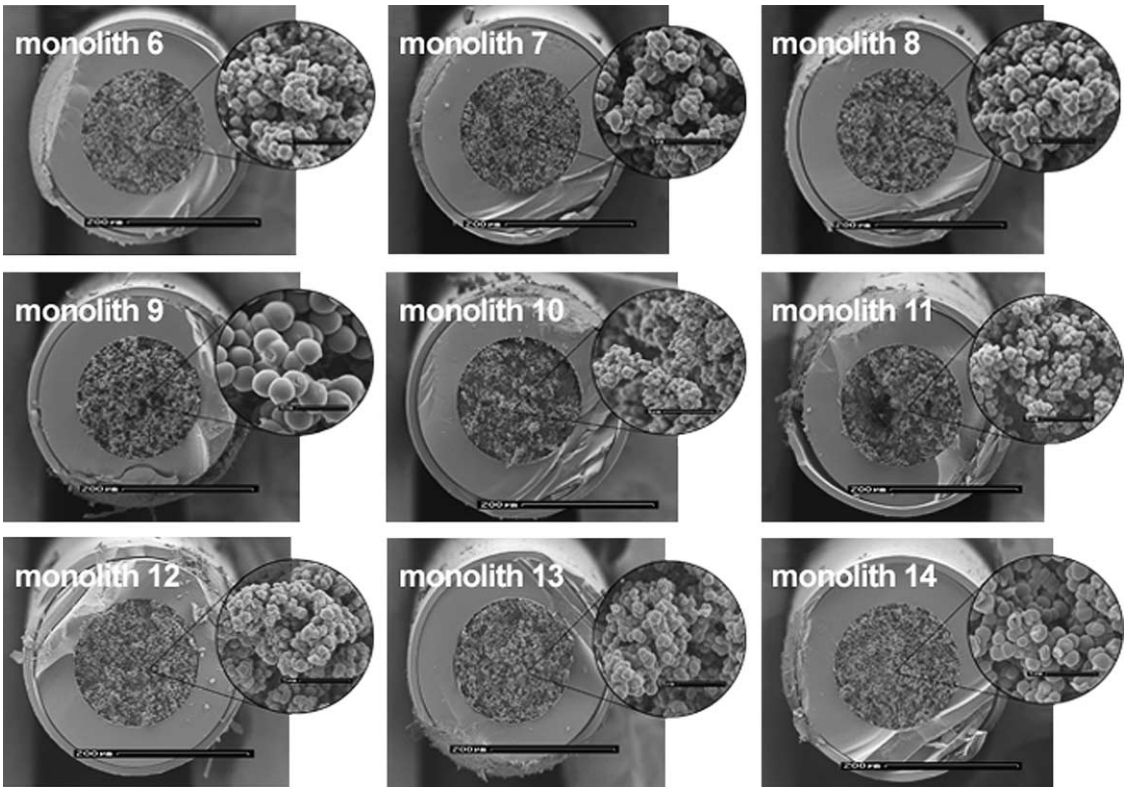


Fig. 5. Electron micrographs of monoliths **6–14**. The scale bar corresponds to 200 μm (5 μm in the inset). For their synthesis, refer to Table 2.

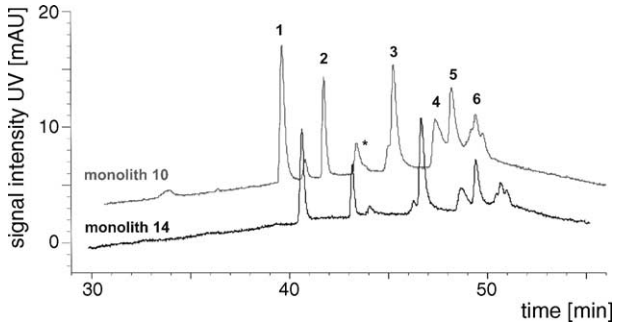


Fig. 7. Separation of the protein standard on monoliths **10** and **14**. (1) Ribonuclease A, (2) insulin, (3) lysozyme, (4) albumin, (5) myoglobin and (6) ß-lactoglobulin, *ghost peak, 2–8 ng of each protein. Chromatographic conditions: mobile phase: (A) 95% water, 5% acetonitrile, 0.05% TFA, (B) 20% water, 80% acetonitrile, 0.04% TFA; gradient: 0–30 min 0–60% (B); 60–90% (B) within 5 min; flow 1 μl/min; 25 °C; detection, UV 190 nm.

the onset of phase separation and is therefore important for the final microstructure. In previous investigations we found that a constant ratio of NBE to DMN-H6 of 1:1 (by weight, corresponding to a molar ratio of 6:4) represents an optimum in terms of separation efficiency of the resulting monoliths [42,43]. We therefore kept the ratio of NBE to DMN-H6 as well as the toluene content constant (1: 1 and 10%, respectively) and solely performed variations in the NBE plus DMN-H6 content. Investigations by means of electron microscopy revealed an increase in microglobule diameter from 0.4 to 1.2 μm resulting from an increase in the NBE plus DMN-H6 content from 20 to 60 wt.% (Fig. 5, monolith **10**–**14**). While these changes in morphology did not have any significant influence on the separation of proteins (Fig. 7), there is an influence on the separation of the homologous insulin's (data not shown) and the peptide standard (Fig. 8).

Thus, t_R increased with an increased NBE plus DMN-H6 content. This was not surprising, since the total amount of stationary phase increased. However, this effect was again only observed for low molecular weight analytes, which have

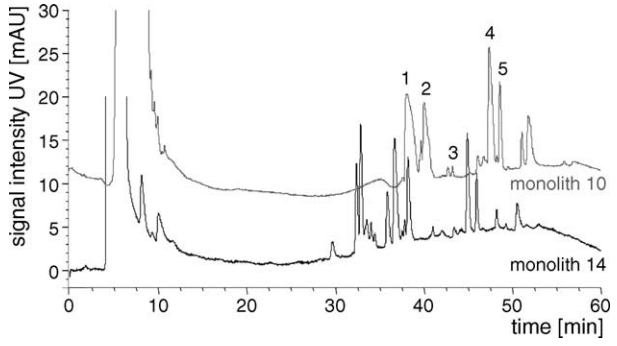


Fig. 8. Separation of the tryptic digest of cytochrome C on monoliths **10** and **14**. (1)–(5) fragments, 120 ng of cytochrome C. Chromatographic conditions: mobile phase: (A) 95% water, 5% acetonitrile, 0.05% TFA, (B) 20% water, 80% acetonitrile, 0.04% TFA; gradient: 0–30 min 0–50% (B); 50–90% (B) within 5 min; flow 1 μl/min; 25 °C; detection, UV 190 nm.

a stronger interaction with micropores. Large analytes tended to show reduced retention times due to reduced values for ε_t.

3.3.3. *Influence of the monomer content on back pressure*

Clearly, the increase in packing density by applying higher monomer (i.e. NBE + DMN-H6) amounts is reflected by an increase in back pressure. Furthermore, a higher variability of the relative standard deviation of the slope *k* (plotting column pressure versus flow rate) with increasing amounts of monomer plus crosslinker was observed. As already discussed above, ROMP is an exothermic process. Therefore, by increasing the content of monomer in the polymerization mixture a higher temperature gradient is built-up within the monolith. As a consequence, reproducibility was decreased with increasing monomer plus crosslinker content. Thus, the relative standard deviation of the slope *k* increased from 25% for monoliths based on 50% monomer plus crosslinker content (monoliths **1**–**5**) to 45% for monoliths based on 60% monomer plus crosslinker content (monoliths **14a**–**c**). Though 25% appears rather high, this is acceptable in view of the good reproducibility in chromatographic separations. However, the relative standard deviation of the slope can be reduced by cooling capillary monoliths during polymerization process.

Comparing the results of previous studies on the effect of changes in polymerization parameters performed with analytical columns [52] to capillary columns, identical trends on separation performance were observed. Nevertheless, monoliths in the capillary format showed significant differences in microglobule shape (Fig. 9).

This change in morphology most probably results from differences in polymerization kinetics. Due to the comparably low polymerization volume and the higher surface-to-volume ratio of the capillaries an improvement in temperature control during the polymerization process was observed, resulting in more homogeneous monolithic beds.

3.4. *Loading study*

In terms of the maximum loading, separations of the insulin/albumin standard using different amounts of analytes were carried out. Baseline separation of human and bovine insulin was observed for a concentration of 2.4 ng for each homologous insulin and 50 ng for albumin.

Overloading is usually detected by plotting the peak width $w_{1/2}$ versus the concentration of the analyte. Any non-linear behavior is indicative for overloading. Chromatographic investigations revealed, that for monolith **17** (composed of 20% monomer plus crosslinker content), the amount of albumin had to be <12 ng to avoid peak broadening. For monolith 20 (composed of 50% monomer plus crosslinker content) the amount of albumin had to be <50 ng (Fig. 10). Clearly, the higher loading capacity correlates with an increase in monomer content.

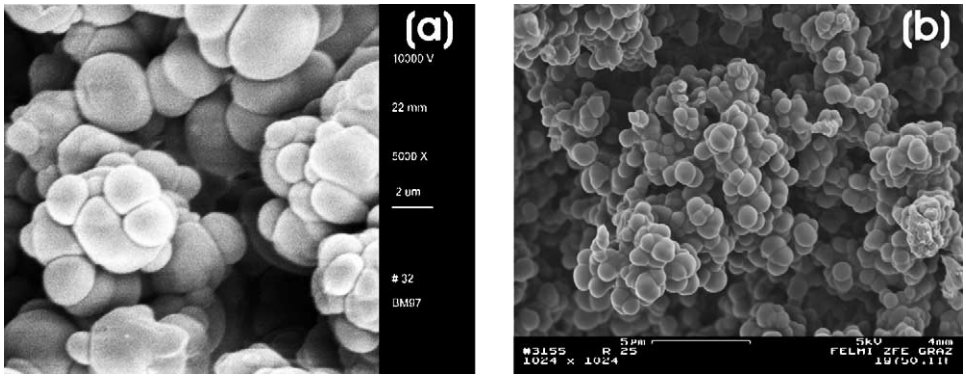


Fig. 9. Electron micrographs of (a) analytical monolith 3 mm i.d. borosilicate glass column (reprint in part with permission from [47]. Copyright (2005) American Chemical Society), scale bar corresponds to 2 μm and (b) capillary monolith 200 μm i.d. fused silica column, scale bar corresponds to 5 μm.

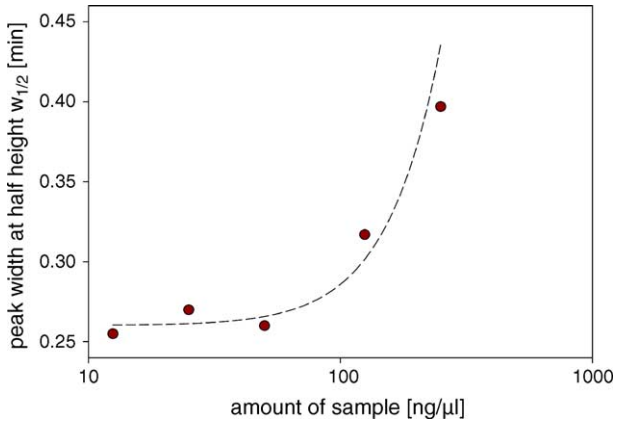


Fig. 10. Loading study on monolith **20** using albumin, 12.5–250 ng. Chromatographic conditions: mobile phase: (A) 95% water, 5% acetonitrile, 0.05% TFA, (B) 20% water, 80% acetonitrile, 0.04% TFA; gradient: 0–30 min 29–33% (B); 33–90% (B) within 5 min; flow 1 μl/min; 25 °C; detection, UV 190 nm.

3.5. Breakthrough-curves

For the exact determination of loading capacities a series of breakthrough curves was recorded. A representative chromatogram is shown in Fig. 11.

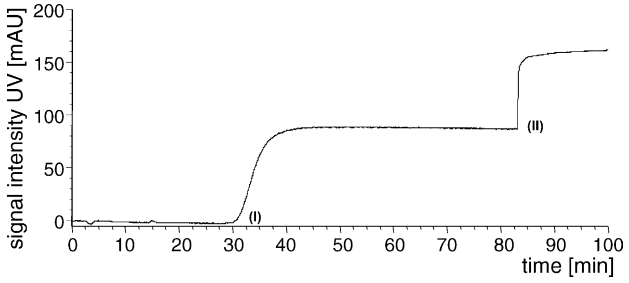


Fig. 11. Breakthrough curve for albumin recorded on monolith **18**. (I) change of 100% mobile phase A to 100% mobile phase B, (II) point of breakthrough. Chromatographic conditions: mobile phase: (A) 95% water, 5% acetonitrile, (B) 95% water, 5% acetonitrile, 0.05% TFA, 0.25 mg/ml BSA; flow 1 μl/min; 25 °C; detection, UV 190 nm.

"I" indicates the changes of solvent A (without TFA) to solvent B (with TFA) and therefore the onset of loading. Albumin was loaded up to the point of breakthrough (II).

Summarizing the data obtained for monoliths **15**–**16** and **18**–**19** (for their compositions refer to Table 2), maximum loading capacities of albumin of 30 ng (RSD 6.9%) and 124 ng (RSD 9.7%) (data obtained at 5% of breakthrough), respectively, was observed. Clearly, the latter possessed higher loading capabilities, a direct consequence of the enhanced amount of stationary phase. At this point, a comparison with other existing stationary phases appears suitable. Oberacher et al. reported on loading capacities of 23 ng of catalase (MW 57 600) for PS-DVB monoliths (60 mm × 0.2 mm capillary columns) [35]. Clearly, the present system possesses capacities similar to these columns.

3.6. Long-term stability

In order to guaranty consistent separation performance, the stability of stationary phases is a major demand in HPLC applications. Any degradation or deterioration of the stationary phase during analysis results in loss of separation efficiency. Earlier studies on stability (DSC-TGA-MS investigations) indicated oxidation process at the vinylene or/and the allylic positions [52], leading to aging. To evaluate long-term stability of norbornene-based capillary columns, consecutive separations of the protein standard were performed at ambient temperature. Trying to reduce time consuming studies to a minimum yet produce relevant data, a period of 3 weeks (150 runs, respectively) was chosen. By comparing the separation parameters of the first and last run of the experiment no significant alterations in separation performance were observed (data not shown).

4. Conclusions

ROMP-derived capillary HPLC columns based on NBE and DMN-H6 have been prepared and characterized in terms of reproducibility in synthesis, influence of selected poly-

merization parameters on chromatographic performance and loading capacity. In summary, reproducibility in synthesis was excellent (1–2% relative standard deviation in t_R). Any change in the polymerization setup had a strong influence on the physico-chemical properties, which directly translate into separation efficiencies. The addition of PPh_3 facilitated handling and had no influence on morphology up to a concentration of 40 ppm. A higher monomer/crosslinker content resulted in improved separation efficiencies for peptides. In parallel, back pressure and loading capacities increased. For capillary dimensions of 140 mm × 0.2 mm i.d., a maximum loading capacity of 30–125 ng for bovine albumin was found. This is comparable to other monolithic systems. Separation properties may reproducibly be varied by variations in monolith recipe. In comparison with published data on semi-preparative monolithic systems (≥3 mm i.d.), similar respectively identical trends were observed. Long-term stability was good at ambient temperature.

Acknowledgements

This study was co-funded by the Styrian Government (NanoTic FA 14C17-50/02-185) and the Austrian Federal Ministry for Transport, Innovation and Technology within the FTSP program. The Austrian Nano-Initiative co-financed this work as part of the Nano-Health project (no. 0200), the sub-project NANO-LITH being financed by the Austrian FFF (Forschungsförderungsfond für die gewerbliche Wirtschaft) (Project no. 252). FMS likes to thank Franz Stelzer from the Technical University of Graz (Austria) for his support. MRB thanks the FWF (Vienna, Austria) for generous financial support (START Y-158).

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