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Ring-opening metathesis polymerization-derived monolithic capillary columns for high-performance liquid chromatography Downscaling and application in medical research

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Abstract

Monolithic capillary columns were prepared via ring-opening metathesis polymerization (ROMP) using norborn-2-ene (NBE) and 1, 4, 4a, 5, 8, 8a-hexahydro-1, 4, 5, 8-*exo*,*endo*-dimethanonaphthalene (DMN-H6) as monomers. The monolithic polymer was copolymerized with Grubbs-type initiator $RuCl_2(PCy_3)_2(CHPh)$ and a suitable porogenic system within the confines of fused silica capillaries of different inner diameter (I.D.). The first part of the study focused on batch-to-batch reproducibility of ROMP-derived capillary monoliths. Capillary monoliths of 200 μm I.D. showed good reproducibility in terms of retention times, with relative standard deviations (RSD) of 1.9% for proteins and 2.2% for peptides. However, the separately synthesized capillary monoliths revealed pronounced variation in back pressure with RSD values of up to 31%. These variations were considerably reduced by cooling of the capillaries during polymerization. Using this optimized preparation procedure capillary monoliths of 100 and 50 μm I.D. were synthesized and the effects of scaling down the column I.D. on the morphology and on the reproducibility of the polymerization process were investigated. In the second part, the applicability of ROMP-derived capillary monoliths to a separation problem common in medical research was assessed. A 200 μm I.D. monolithic column demonstrated excellent separation behavior for insulin and various insulin analogs, showing equivalent separation performance to Vydac C4 and Zorbax C3-based stationary phases. Moreover, the high permeability of monoliths enabled chromatographic separations at higher flow rates, which shortened analysis time to about one third. For the analysis of insulin in human biofluid samples, enhanced sensitivity was achieved by using a 50 μm I.D. ROMP-derived monolith.

Keywords: Monolith; Metathesis polymerization; High-performance liquid chromatography (HPLC); Capillary columns; Proteins; Peptides; Insulin

1. Introduction

Speed of analysis and sensitivity are of great importance in medical research. To keep pace with the ever-increasing demands of analytical science, there is a constant need for further development and improvement in instrumental analysis. At present, the state of the art instrumentation setup in medical research is high-performance liquid chromatography (HPLC) in combination with mass spectrometric (MS) detection. While MS technology has made considerable progress during the past years, the successful separation of analytes prior to MS detection still remains a bottleneck in analysis [1]. Beyond the advances in instrumentation in chromatographic science, it is the progress in column technology that possesses great potential for improvements in separation power and speed. For more than 30 years, particle-packed columns have been the separation media of choice for chromatographic techniques. However, the limitations of particulate, porous stationary phases in the field of bioanalysis, such as slow diffusional mass transfer for large molecules [2,3] and the large interstitial void volume, have called for further developments [4]. Several attempts have been made to

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overcome these limitations and to enhance separation efficiency and shorten analysis time. This includes, for example, the implementation of perfusion particles [5–8], the elimination of diffusive pores [9–11] and/or the use of smaller particles [12–14], whereas the latter is accompanied by elevated back pressures and, therefore, the need for ultra high pressure systems (UPLC).

Another approach involves monolithic separation media showing completely different stationary phase characteristics as monoliths consist of one single piece of highly porous material. At present, several reviews are available, focusing on the different types of monolithic material whether based on inorganic silica [4,15,16] or organic polymer [17–19], addressing both monolith characteristics and their applications. Guiochon [20] certainly provided the most comprehensive overview in this field and showed that monoliths have undergone a long evolution since their introduction into the scientific community. However, till now the number of applications using monolithic columns still remains much smaller compared to packed columns, which can be related to limited commercial availability [21] and issues of batch-to-batch reproducibility [22]. Nevertheless, monolithic separation media are gaining more and more attention in chromatographic science due to their beneficial properties. Their high permeability enables high flow rates at comparable low back pressure, making them ideally suited for high-throughput separations. Furthermore, the simple preparation procedure, without the requirement of sophisticated packing procedures or the manufacturing of end frits allows for the preparation of different column dimensions with almost no limitation in column diameter. To date, one of our contributions in this field was the introduction of a new type of monolithic, organic stationary phase prepared by ring-opening metathesis polymerization (ROMP) [23,24] and the preparation of monolithic columns ranging from 5 cm to 200 μm inner diameter (I.D.) [25–32].

Continuing our work, we report here on a further downscaling of ROMP-derived monoliths down to 50 μm I.D. First of all, the reproducibility of the synthesis of 200 μm I.D. monoliths was studied and an optimization of the preparation procedure in order to enhance reproducibility was performed. Using this optimized preparation procedure the downscaling process was investigated in terms of the reproducibility of column preparation and changes in morphology. To show the applicability of capillary monoliths for medically relevant analytes, separation of insulin and various insulin analogs were performed. To show the competitiveness of monoliths, a comparison of monolithic separation media with particle-packed stationary phases typically used in protein and peptide analysis was conducted. Furthermore, the potential of a 50 μm I.D. ROMP monolith for the separation of insulin in human biofluid samples was investigated.

2. Experimental

2.1. Chemicals, reagents and samples

[2.2.1]Bicyclohept-2-en-5-ylmethyldichlorosilane (95%) was purchased from ABCR (Karlsruhe, Germany). NaOH (99%), acetone (99.0%), toluene secco solv. and 2-propanol (2-PrOH) secco solv. were purchased from Merck (Darmstadt, Germany). Pyridine (99%) was purchased from Pierce (Rockford, IL, USA), [2.2.1]bicyclohept-2-ene (norborn-2-ene, NBE) (97%) from Fluka (Buchs, Switzerland). Methanol (99.7%) and acetic acid (99%) were purchased from Riedel-de Haën (Seelze, Germany). 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) according to the literature [33]. Ethyl vinyl ether (99%), $RuCl_2(PCy_3)_2(CHPh)$ (Cy = cyclohexyl) (97%), triphenylphosphine (PPh_3) (99%), water (HPLC-grade), acetonitrile (HPLC-grade), trifluoroacetic acid (TFA) (99.5%), ribonuclease A (from bovine pancreas), lysozyme (from chicken egg white), albumin (from bovine serum), myoglobin (from horse skeletal muscle), β-lactoglobulin (from bovine milk), human insulin (recombinant expressed in yeast), bovine insulin (from bovine pancreas), porcine insulin (from porcine pancreas) and the peptide separation buffer (PSB) were purchased from Sigma–Aldrich (Vienna, Austria). All peptides e.g. met-enkephalin, angiotensin III, luteinizing hormone-releasing hormone (LHRH), neurotensin, bombesin, and substance P were purchased from Bachem (Bubendorf, Switzerland) with the exception of leu-enkephalin, which was purchased from Fluka. All standards were prepared by dissolving them in peptide separation buffer, followed by dilution with mobile phase and were stored at −80 °C. Insulin glargine (Sanofi-Aventis, Vienna, Austria), insulin lispro (Lilly, Vienna, Austria) and insulin aspart (Novo Nordisk, Vienna, Austria) were purchased as injection solutions.

2.2. Synthesis of ROMP-based monolithic capillary columns

Fused silica capillaries (non-deactivated, 365 μm O.D., 200, 100 and 50 μm I.D.), were purchased from J & W (Agilent, Palo Alto, CA, USA). Surface modification of the capillaries was performed as described previously [34]. Synthesis of monoliths was accomplished using a published protocol with NBE, DMN-H6, toluene, 2-propanol and $RuCl_2(PCy_3)_2(CHPh)$ [28]. Polymerization of capillary monoliths was performed at 25 °C or at 0 °C using an ice bath. Table 1 summarizes the polymerization parameters (composition and polymerization temperature) and column dimensions of all monoliths used in this study. The length of the monolithic capillary columns was 15 cm, unless stated otherwise. Electron microscopy was carried out at the Center for Electron Microscopy, ZFE, Graz, Austria.

2.3. High-performance liquid chromatography–UV

The HPLC system for chromatographic evaluation of batch-to-batch reproducibility consisted of a Beckman P 125 HPLC pump (Beckman Coulter, Inc., Fullerton, CA, USA), an Acurate Pre-Column Flow Splitter (Dionex, LC-Packings, Amsterdam, The Netherlands), a Triathlon autosampler (Spark Holland, Emmen, The Netherlands), a column oven STH 585 (Dionex, Sunnyvale, CA, USA) and an Ultimate UV-detector (Dionex, LC-Packings). The separation of insulin using downscaled

Table 1
Composition and polymerization temperature of all monoliths considered in this study[a]

Monolith number	Column inner diameter (μm)	NBE (%, (w/w))	DMN-H6 (%, (w/w))	2-PrOH (%, (w/w))	Toluene (%, (w/w))	Polymerization temperature (°C)
1–5	200	25	25	40	10	25
6–10	200	25	25	40	10	0
11–13	200	10	10	70	10	25
14–16	200	10	10	70	10	0
17–19	100	10	10	70	10	0
20–22	50	10	10	70	10	0
23–25	100	10	10	70	10	25
26–28	50	10	10	70	10	25
29	200	20	20	50	10	0

[a] The concentration of initiator was 0.5% (wt.%) and 40 ppm Ph_3P as moderator was used throughout.

monoliths was performed on an Ultimate capillary HPLC system (Dionex, LC-Packings) comprising a Triathlon autosampler (Spark Holland). The UV detection cell volume was 3 nl in all cases. Data acquisition was accomplished using Chromeleon software (Version 6.40).

2.4. High-performance liquid chromatography–mass spectrometry

All experiments were performed on an Ultimate capillary HPLC system (Dionex, LC-Packings) coupled to a ThermoFinnigan Quantum TSQ Ultra AM (Spectronex, Basel, CH). The system was controlled by Xcalibur-Software 1.3. For the comparison of the separation performance of a capillary ROMP monolith with particle-packed capillary columns (250 × 0.3 mm, 5 μm particle diameter), Vydac C4 (Dionex, LC-Packings) and Zorbax C3 (Agilent) capillary columns were used.

Positive ESI-MS mass spectrometry was performed using nanospray at 2000 V and a transfer capillary temperature of 200 °C. All insulins were detected as +5 charged molecular ions e.g. bovine insulin (MW 5733 g/mol, $m/z = 1147.6$), porcine insulin (MW 5778 g/mol, $m/z = 1155.5$), human insulin and insulin lispro (MW 5807 g/mol, $m/z = 1162.4$), insulin aspart (MW 5825 g/mol, $m/z = 1166.4$) and insulin glargine (MW 6063 g/mol, $m/z = 1213.6$).

3. Results and discussion

3.1. Preparation of capillary monoliths

A basic requirement for the application of a stationary phase in LC is reproducible column production in order to guarantee constant separation characteristics. As far as the production of 200 μm I.D. capillary monoliths via ROMP is concerned, batch-to-batch reproducibility has previously been demonstrated [34]. In this publication, good reproducibility of chromatographic parameters (e.g. retention times t_R, peak with at half height $w_{1/2}$, symmetry S and resolution R) for protein and peptide separation was reported. However, notable batch-to-batch variations in back pressure were observed. We assumed that these variations could be attributed to insufficient temperature control during the exothermic polymerization process leading to an inhomogeneous monolithic structure.

3.1.1. Reproducibility of capillary monolith preparation

In order to improve batch-to-batch reproducibility, we tried to dissipate the exothermic reaction heat more homogenously by cooling the capillaries during monolith formation. To investigate the influence of the polymerization temperature on reproducibility and morphology, we chose a monolith composition showing high variations in back pressure [34]. Overall, five monoliths were prepared at 25 °C (**1**–**5**) and 0 °C (**6**–**10**) and were subjected to chromatographic testing using a mixture of six model proteins (i.e. ribonuclease A, human insulin, lysozyme, bovine albumin, myoglobin and β-lactoglobulin) and a peptide standard consisting of met-enkephalin, leu-enkephalin, angiotensin III, LHRH, neurotensin, bombesin, and substance P. Two parameters were chosen to evaluate the reproducibility of column preparation: (i) the retention times of proteins and peptides and (ii) the back pressure, which directly reflects the hydrodynamic properties of the monolithic bed.

As a self-made capillary (cap)-HPLC with an external flow splitting unit was used, run-to-run precision of the cap-HPLC system was checked prior to column evaluation. Threefold, consecutive injections of each standard system were performed and the relative standard deviation (RSD) of t_R was within specifications [35], with 0.1–0.7% for the protein standard. The values of run-to-run precision for the peptide standard were slightly higher (1.0–1.4%), which can be attributed to the flat solvent gradient used. Nevertheless, the values were still acceptable for the cap-HPLC setup used.

Chromatographic evaluation of batch-to-batch reproducibility for monoliths prepared at 25 °C revealed good reproducibility in terms of t_R with a mean RSD of 1.9% and 2.2% (including run-to-run precision) for proteins and peptides, respectively. These findings are consistent with previously published results [34]. Despite our assumption capillary monoliths polymerized at 0 °C showed only a slight improvement in variation of t_R to 1.5% RSD for proteins and 2.0% RSD for peptides. However, cooling of the capillaries during the polymerization process clearly had an influence on separation performance of monoliths. As shown in Table 2, an increase in t_R was observed for both standard systems. Moreover, the performance of peptide separation was

Table 2
Retention times t_R of protein and peptide separations performed on monoliths (150 mm × 0.2 mm) polymerized at 25 °C (**1–5**) and 0 °C (**6–10**). Values of RSD represent the sum of run-to-run precision and batch-to-batch reproducibility

	Monolith number					Mean t_R	RSD	Monolith number					Mean t_R	RSD
	1	2	3	4	5	(min)	(%)	6	7	8	9	10	(min)	(%)
Proteins[a]														
Ribonuclease A	25.66	25.44	25.63	25.74	24.74	25.44	1.61	26.06	25.57	25.40	25.23	25.26	25.50	1.33
Insulin	27.90	27.64	27.88	28.06	26.86	27.67	1.72	28.58	27.96	27.86	27.84	27.76	28.00	1.18
Lysozyme	31.84	31.45	31.68	32.14	30.61	31.54	1.84	32.96	31.72	31.71	31.38	31.45	31.84	2.02
Albumin	34.01	33.68	33.86	34.26	32.73	33.71	1.74	35.26	34.31	34.16	33.75	-	34.37	1.85
Myoglobin	35.19	34.70	34.97	35.57	33.57	34.80	2.17	35.96	34.98	35.00	34.57	34.61	35.02	1.59
β-Lactoglobulin	36.49	36.48	36.31	37.38	35.23	36.38	2.11	37.28	36.69	36.41	36.69	36.11	36.64	1.17
Peptides[b]														
Met-enkephalin	18.62	18.56	18.61	18.98	17.68	18.49	2.61	19.98	19.39	18.86	18.75	18.56	19.11	3.01
Leu-enkephalin	20.00	19.86	19.93	20.36	18.97	19.82	2.59	21.30	20.83	20.26	20.22	19.94	20.51	2.67
Angiotensin III	[c]	[c]	[c]	[c]	[c]	–	–	21.73	–	21.29	20.97	20.87	21.22	1.84
LHRH	21.16	21.05	20.95	21.40	20.27	20.97	2.02	22.55	22.12	21.73	21.41	21.32	21.83	2.35
Neurotensin	23.21	23.12	23.04	23.50	22.22	23.02	2.08	23.67	24.11	23.66	23.23	23.15	23.56	1.65
Bombesin	24.28	24.17	24.11	24.58	23.28	24.08	2.00	24.93	25.25	24.96	24.47	24.37	24.80	1.48
Substance P	24.81	24.66	24.70	25.23	23.74	24.63	2.22	25.45	25.84	25.41	24.82	24.81	25.27	1.76

Chromatographic conditions: mobile phase: (A) 95% water, 5% acetonitrile, 0.05% TFA, (B) 20% water, 80% acetonitrile, 0.04% TFA.
[a] Gradient: 0–30 min 0–60% B; 60–90% B within 5 min; flow 1 μl/min; 25 °C; detection, UV 190 nm, inj.: 2–10 ng of each protein.
[b] Gradient: 0–30 min 0–50% B; 50–90% B within 5 min; flow 1 μl/min; 25 °C; detection, UV 190 nm, inj.: 1 ng of each peptide.
[c] Coelutes with LHRH.

significantly improved. Fig. 1 illustrates the chromatographic separations of peptides performed on monoliths prepared at different polymerization temperatures, with the monolith polymerized at 0 °C showing significantly enhanced resolution of angiotensin III and LHRH.

Both the improvement in variation of t_R and in separation performance demonstrate that the polymerization temperature has an effect on morphology. In order to confirm this observation, the theory of organic polymer monolith formation must be consulted. This states that the permanent porosity of monolithic beds is created upon phase separation of the insoluble polymer from the porogens during polymerization [36]. For monoliths, as for resins in general, size and morphology of the pores strongly depend on several factors, including solubility of the porogens for the resulting polymer and polymerization kinetics. Cooling during the polymerization process does have an influence on both factors and therefore, significantly affects microglobe and pore formation [32]. To investigate the changes in morphology of ROMP-derived monoliths electron micrographs were taken. As seen in Fig. 2, no change in microglobe diameter (1.6 ± 0.2 μm and 1.7 ± 0.1 μm for monolith **1** and **6**, respectively) was observed. However, regarding monolith **6** the electron micrographic pictures revealed a more homogeneous distribution of the macro-pores.

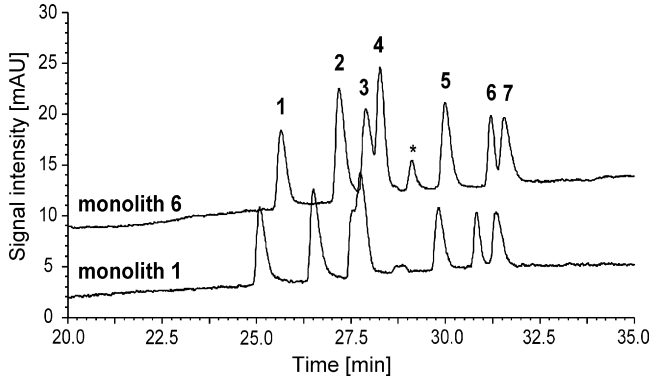

Fig. 1. Separation of the peptide standard on monoliths 1 and 6. (1) Met-enkephalin, (2) leu-enkephalin, (3) angiotensin III, (4) LHRH, (5) neurotensin, (6) bombesin and (7) substance P, *ghost peak, 1 ng of each peptide. Monolith dimensions: 150 mm × 0.2 mm; chromatographic conditions: mobile phase: (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.

In order to support the assumption that cooling the capillaries during polymerization unifies the porous characteristics of ROMP-derived monoliths, the hydrodynamic properties were investigated. Consistent with our previously published results [34] monoliths polymerized at 25 °C showed rather high variations in back pressure. However, the variability of the slope k of 31% RSD was decreased to 8% while setting the polymerization temperature to 0 °C. Therefore, the improvement in variation of t_R and of hydrodynamic properties clearly reveal the elimination of irregularities in porous distribution when the exothermic reaction heat is efficiently dissipated.

To verify the hypothesis that reaction heat strongly influences batch-to-batch reproducibility of ROMP-derived capillary columns, the monomer content of the polymerization mixture was decreased. A lower weight percentage of monomers leads to less exothermic reactions during monolith formation. According to our theory, this would significantly improve batch-to-batch reproducibility. Monoliths comprising of 20 wt.% of monomer were prepared at 25 °C (**11–13**) and 0 °C (**14–16**) and tested in terms of hydrodynamic properties. In comparison to monoliths based on 50 wt.% of polymer, a significant decrease in variations of the slope k was observed, with 17% and 5% RSD for

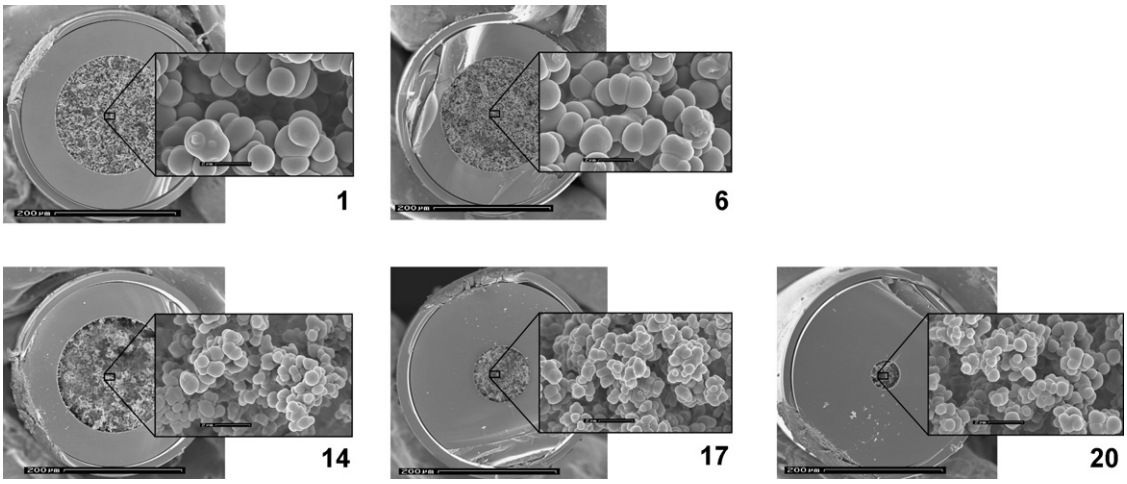

Fig. 2. Electron micrographs of monoliths **1**, **6**, **14**, **17** and **20**. The scale bar corresponds to 200 μm (2 μm in the insert).

monoliths polymerized at 25 °C and 0 °C, respectively, demonstrating that the exothermic reaction heat mainly contributes to the variations of separately synthesized monoliths.

3.1.2. Downscaling of column inner diameter

To date, ROMP-derived monolithic stationary phases have been prepared and characterized with varying geometry ranging from analytical to capillary dimensions. However, scaling down the column I.D. influences the resulting monoliths in terms of structure. Downscaling from 3 mm to 200 μm I.D. revealed significant differences in morphology and column performance [31]. After characterization of 200 μm I.D. ROMP-derived monoliths, we took a further step with the preparation of monoliths within the confines of 100 μm (**17**–**19**) and 50 μm (**20**–**22**) I.D. fused silica capillaries. Using the last polymer composition all monoliths were polymerized at 0 °C in order to guarantee best homogeneity of the resulting polymer. The effects of scaling down the column I.D. on the resulting monolithic morphology and on reproducibility of the polymerization process were investigated.

Electron microscope pictures of monoliths **14**, **17** and **20** are shown in Fig. 2 and revealed no significant difference in morphology for capillary monoliths of 200, 100 and 50 μm I.D. Monolithic microglobe diameter was identical for all column dimensions. However, as electron micrographs do not completely characterize morphological differences, the hydrodynamic properties were evaluated. Particular attention was given to reproducibility of the preparation process. By plotting column back pressure versus linear flow velocity, the variability of the slope *k* increased from 5 to 13% RSD when column I.D. was decreased from 200 to 50 μm. These results were unexpected as scaling down of the column I.D. should have a positive effect on reproducibility. Due to the lower polymerization volume and the increasing surface-to-volume ratio, the reaction heat of the exothermic polymerization should be even more efficiently dissipated when decreasing the column I.D. In order to investigate this effect, we additionally prepared monoliths at 25 °C (**23**–**28**). The increase in variability was even more pronounced with a range from 17% (200 μm I.D.) to 32% (50 μm I.D.). Hence, while decreasing the column I.D. one must deal with two interfering effects. As outlined earlier, there are (i) fewer irregularities in pore distribution due to the more efficient heat dissipation. However, (ii) due to the increasing ratio of pore size to monolithic cross-section, these irregularities have a marked effect on hydrodynamic properties. Nevertheless, for monolithic columns of different I.D. polymerized at 0 °C almost no difference in column back pressure was observed (Fig. 3). All monoliths showed linear behavior up to 100 mm/s. Even at this high flow velocity the back pressure for 50 μm I.D. monoliths did not exceed 18 MPa.

3.2. Monoliths in medical research

Up to this point, we predominantly used artificial mixtures to characterize and optimize ROMP-derived monoliths for their use in bioanalysis. Based on these promising results we now demonstrate their applicability in medical research. We therefore chose an ambitious analytical challenge of medical importance, with the separation of insulin and various insulin analogs being therapy of choice for the treatment of diabetes. During the last decade, new insulin analogs have been developed with pharma-

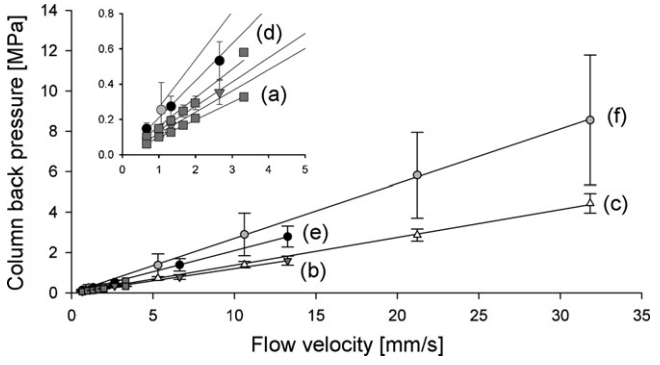

Fig. 3. Column back pressure vs. linear flow velocity for monoliths of (a) 200 μm, (b) 100 μm and (c) 50 μm I.D. polymerized at 0 °C, (d) 200 μm, (e) 100 μm and (f) 50 μm I.D. polymerized at 25 °C. The length of monoliths was 10 cm throughout. Mobile phase: water.

cokinetic profiles differing from existing insulin preparations [37] such as short-acting analogs including insulin lispro and aspart, and long-acting insulin glargine. The high structural resemblance of insulin to its analogs and the low analyte concentration are putting high demands on analytical techniques. Due to the high cross-reactivity of the insulins the simultaneous determination and quantification is not possible by enzyme-linked immunosorbent assays. Therefore, HPLC/MS analysis represents a powerful tool.

3.2.1. Monolith versus particle-packed columns

To investigate the potential of ROMP-derived monoliths for insulin separation, a direct comparison with particle-based stationary phases typically used in peptide analysis was performed. For this purpose, we selected monolith **29** as it showed best performance for this particular task. A mixed standard of human insulin and various insulin analogs used in diabetic treatment, e.g. bovine insulin, porcine insulin, insulin lispro, insulin aspart and insulin glargine was employed. Human insulin and insulin lispro exhibit the same amino acid composition, however, the last two amino acids of the B-chain are switched in position. Thus, they have the same mass and are not distinguishable by MS. Therefore, their chromatographic separation is a key factor in simultaneous insulin analysis. In order to obtain the best separation, the gradient was marginally adopted separately for each stationary phase whereas the flow velocity was kept constant at 2 mm/s. Fig. 4 shows the separation of the mixed insulin standard performed on a Vydac C4, Zorbax C3 and a ROMP-derived monolithic capillary column. Although only minor changes in separation conditions were performed, all stationary phases showed similar separation behavior suggesting that they are nearly comparable in terms of hydrophobicity. The Vydac C4 capillary column demonstrated best resolution (0.94) of human insulin and insulin lispro followed by ROMP monolith and Zorbax C3 (0.71 and 0.57, respectively). However, when applying higher flow rates the superiority of the monolith becomes apparent.

Shorter separation times, leading to a reduction in costs, are an important factor in medical analysis. Taking advantage of the high permeability of the monolithic capillary column, insulin separation was performed at 25 μl/min (20 mm/s) which is not feasible on the particle-based Vydac C4, Zorbax C3 capillary columns (maximal 10 mm/s recommended). Fig. 5 shows the isocratic elution of insulin bovine, porcine as well as human insulin and insulin lispro within 10 min. Thus, analysis time was reduced to one third while maintaining excellent separation performance.

3.2.2. Application of downscaled capillary monoliths

In medical research, blood is used routinely for quantitative determination of biomolecules for diagnostic purposes due to its advantages of easy access and sample volume in the ml-range. A major disadvantage of blood is that it only distributes substances in the body, whereas the interstitium represents the actual chemical environment of every living cell. The interstitial fluid (ISF) provides a path through which substances must travel between the blood capillaries and the cells. Therefore, analysis of the ISF permits a more precise characterisation of the effect of medical agents and hormones [38]. However, ISF samples place particular demands on analytical techniques, as only low

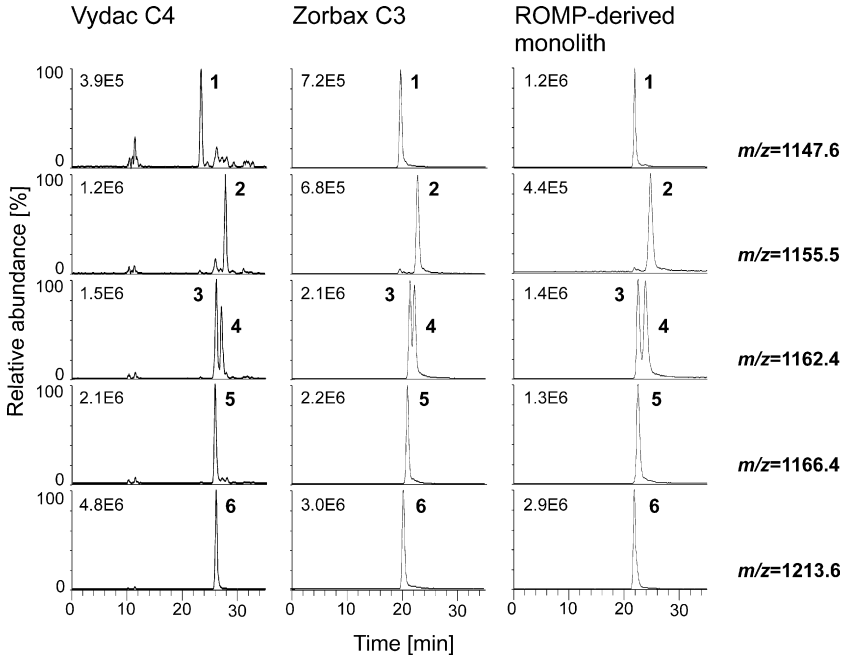

Fig. 4. Separation performance of particle-packed capillary columns (250 mm × 0.3 mm) and a ROMP-derived monolithic column (250 mm × 0.2 mm) for a mixture of insulins containing (1) bovine insulin (2) porcine insulin, (3) insulin lispro, (4) human insulin, (5) insulin aspart, (6) insulin glargine, detected as +5 charged molecular ions, 100–500 fmol each. Chromatographic conditions: mobile phase: (A) 100% water, 1% acetic acid, 0.05% TFA, (B) 40% water, 59% acetonitrile, 1% acetic acid, 0.05% TFA; gradient: (a) 45% B to 50% B within 25 min, flow 4 μl/min, 50 °C; (b) 50% B to 55% B within 25 min, flow 4 μl/min, 50 °C; (c) 43% B to 47% B within 25 min, flow 2.5 μl/min, 25 °C.

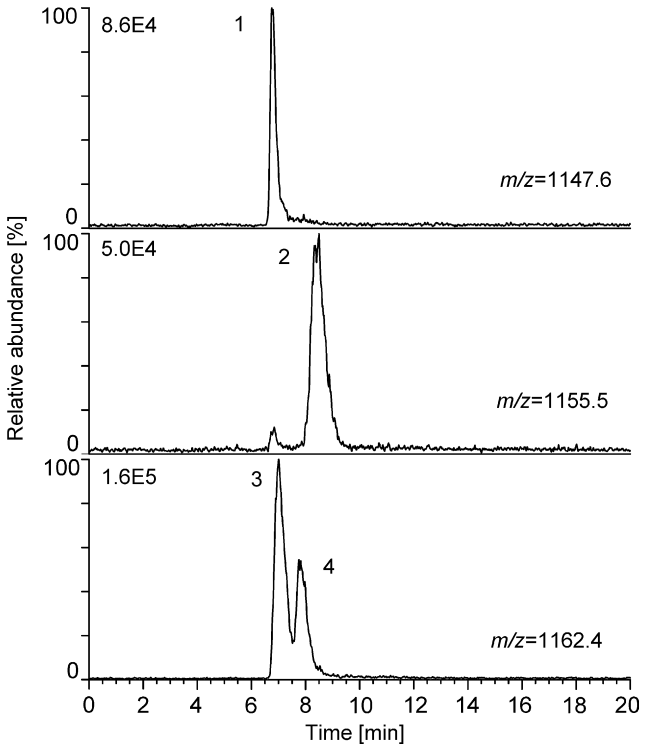

Fig. 5. Isocratic separation of human insulin and insulin analogues using monolith **29**. (1) Bovine insulin, (2) porcine insulin, (3) insulin lispro, (4) human insulin, detected as +5 charged molecular ions, 100–500 fmol each. Chromatographic conditions: monolith dimension: 150 mm × 0.2 mm; isocratic elution using 26% acetonitrile in water containing 0.1% acetic acid and 0.05% TFA, flow 25 μl/min, 25 °C.

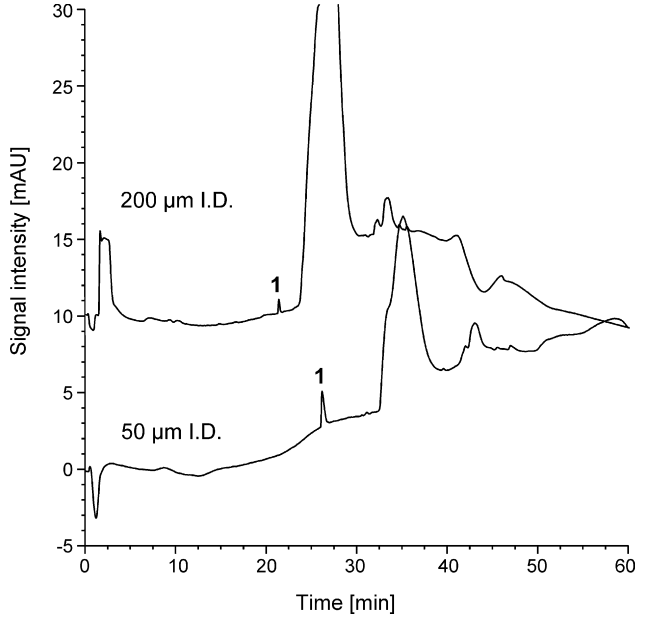

Fig. 6. Analysis of a human insulin in interstitial fluid samples using ROMP-derived capillary monoliths of (a) 200 μm I.D. and (b) 50 μm I.D. Interstitial fluid samples diluted (a) 1:10 and (b) 1:160 and spiked with (1) human insulin (100 fmol/μl), injection volume 1 μl. The length of monoliths was 8 cm throughout. 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; 50–90% B within 5 min; flow (a) 1.5 μl/min, (b) 0.5 μl/min; 25 °C; detection, UV 190 nm.

sample volumes in the μl-range per minute and low analyte concentration are gained. Thus, sensitivity of insulin measurement is important when performing ISF experiments in the field of diabetes. One promising way to achieve sensitive analysis is to reduce the I.D. of the separation column [39].

Using a ROMP-derived capillary monolith of 200 μm I.D. (monolith **2**) an ISF sample spiked with human insulin was analyzed. The monolith demonstrated excellent separation of insulin from the main ISF component albumin, being present at approximately 1%. However, as shown in Fig. 6 the signal intensity was rather low. To enhance the sensitivity of analysis, the experiment was repeated on a 50 μm I.D. monolith (monolith **20**). Although the monoliths were not comparable in their polymer composition, the separation performance remained unaffected. Furthermore, using the downscaled monolithic capillary column a sixfold increase in sensitivity was achieved.

4. Conclusion

The polymerization process for the preparation of ROMP-derived monolithic capillary columns was investigated in order to guarantee the reliable production of capillary columns. Although capillary monoliths already exhibit a high degree of batch-to-batch reproducibility, the reproducibility was further enhanced by a modification of the preparation process. Temperature control during polymerization has a positive effect on homogeneity of the monolithic structure, leading to enhanced reproducibility in terms of back pressure.

In addition to rapid and highly efficient separation of proteins and peptides, we were able to demonstrate the separation power of ROMP-derived monoliths in medical research. For the parallel analysis of insulin and various insulin analogs, ROMP-derived monoliths showed separation performance equivalent with particle-based stationary phases typically used in peptide analysis. However, the unique structural properties of monolithic columns enable fast chromatographic separations at high flow rates, thus enabling a reduction in analysis time. Using ROMP monoliths, separation of an insulin mixture was performed at 25 μl/min, which is not possible with particle-packed capillary columns. Furthermore, scaling down the size of ROMP-derived monoliths offers great potential for their use in medical research, with monolithic columns of 50 μm I.D. showing good separation performance combined with enhanced sensitivity.

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