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Characterization of some physical and chromatographic properties of monolithic poly(styrene–co-divinylbenzene) columns

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Abstract

Monolithic capillary columns were prepared by copolymerization of styrene and divinylbenzene inside a 200 μm i.d. fused silica capillary using a mixture of tetrahydrofuran and decanol as porogen. Important chromatographic features of the synthesized columns were characterized and critically compared to the properties of columns packed with micropellicular, octadecylated poly(styrene–co-divinylbenzene) (PS–DVB–C_{18}) particles. The permeability of a 60 mm long monolithic column was slightly higher than that of an equally dimensioned column packed with PS–DVB–C_{18} beads and was invariant up to at least 250 bar column inlet pressure, indicating the high-pressure stability of the monolithic columns. Interestingly, monolithic columns showed a 3.6 times better separation efficiency for oligonucleotides than granular columns. To study differences of the molecular diffusion processes between granular and monolithic columns, Van Deemter plots were measured. Due to the favorable pore structure of monolithic columns all kind of diffusional band broadening was reduced two to five times. Using inverse size-exclusion chromatography a total porosity of 70% was determined, which consisted of internodule porosity (20%) and internal porosity (50%). The observed fast mass transfer and the resulting high separation efficiency suggested that the surface of the monolithic stationary phase is rather rough and does not feature real pores accessible to macromolecular analytes such as polypeptides or oligonucleotides. The maximum analytical loading capacity of monolithic columns for oligonucleotides was found to be in the region of 500 fmol, which compared well to the loading capacity of the granular columns. Batch-to-batch reproducibility proved to be better with granular stationary phases compared to monolithic stationary phase, in which each column bed is the result of a unique column preparation process.

Keywords: Monolithic columns; Capillary columns; Stationary phases, LC; Poly(styrene–divinylbenzene); Peptides; Nucleic acids

1. Introduction

Stationary phases based on microparticles have been successfully utilized as separation media for high-performance liquid chromatography (HPLC) for almost four decades [1–6]. However, HPLC columns packed with microparticulate, porous stationary phases have some limitations, namely the relatively large void volume between the packed particles and the slow diffusional mass transfer of solutes into and out of the stagnant mobile phase present in the pores of the separation medium, resulting in considerable band broadening particularly with high molecular analytes [7,8]. One possible route to enhance the mass transfer represents the complete elimination of diffusive pores, which restricts the mass transfer to a thin, retentive layer at the outer surface of the stationary phase, resulting in so-called micropellicular stationary phases [9].

An alternative approach to alleviate the problem of restricted mass transfer and intraparticular void volume is the concept of monolithic chromatographic beds, in which the separation medium consists of a continuous rod of a rigid, porous polymer which has no interstitial volume but only internal porosity consisting of micropores and macropores [10–13]. Because of the absence of intraparticular volume, all of the mobile phase is forced to flow through the pores of the separation medium [14]. According to theory, mass transport is enhanced by such convection [15–17], which has a positive effect on chromatographic efficiency. Therefore, monolithic stationary phases have become a rapidly burgeoning field in the preparation of chromatographic stationary phases in recent years [18].

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In general, monolithic columns can be divided into two categories. (i) Silica-based monolithic columns are generally prepared using sol–gel technology. This technology can be applied to create a continuous sol–gel network throughout the column formed by the gelation of a sol solution [13,19,20]. (ii) The second category is represented by rigid organic polymer-based monolithic columns including acrylamide-based [21,22], acrylate- or methacrylate-based [12,23–25], and styrene-based polymers [26–30]. Porous matrices are obtained when polymerization and crosslinking take place in the presence of inert porogens which lead to a phase separation during the ongoing polymerization reaction, effecting the formation of permanent pores in the material.

The concept of monolithic stationary phases is especially favorable for the fabrication of capillary columns [28,31–33]. Covalent immobilization of the monolith at the wall of a fused capillary eliminates the necessity to prepare a tiny retaining frit, which is one of the more tedious and difficult to control steps during the manufacture of packed bed capillary columns [34]. Moreover due to the in situ polymerization of the monolithic chromatographic bed within the confines of a fused silica tube the laborious steps of particle synthesis and column packing could be overcome. On account of this, we introduced monolithic capillary columns prepared by copolymerization of styrene and divinylbenzene inside a 200 μm i.d. fused silica capillary using a mixture of tetrahydrofuran and decanol as porogen for the highly efficient separation of single- and double-stranded nucleic acids by ion-pair reversed-phase HPLC (RP-HPIPC) and of peptides and proteins by reversed-phase HPLC (RP-HPLC) [29,35–37]. Continuing our recent work, a discussion of important physical and chromatographic properties of the monolithic capillary columns is presented in this paper. Characteristics including reproducibility of fabrication, loading capacity, pore size distribution, and molecular diffusion processes within the chromatographic bed were compared to the features of capillary columns packed with micropellicular, octadecylated, 2.1 μm poly(styrene–co-divinylbenzene) particles.

2. Experimental

2.1. Chemicals and samples

Acetonitrile (HPLC gradient-grade), acetic acid (analytical reagent grade), methanol (gradient grade), and water (HPLC grade) were obtained from Merck (Darmstadt, Germany). Trifluoroacetic acid (TFA, for protein sequence analysis), tetrahydrofuran (puriss.), toluene (puriss.), and triethylamine (analytical reagent grade) were purchased from Fluka (Buchs, Switzerland). A 1.0 M stock solution of triethylammonium acetate (TEAA), pH 7.0, was prepared by adding acetic acid to a 1.0 M aqueous solution of triethylamine until pH 7.0 was reached.

Polystyrene standards for size-exclusion chromatography were obtained from Polymer Standards Service (PSS: Mainz, Germany) (mass, 94 650), and from Waters (Milford, MA, USA) (masses, 440, 2350, 3600, 6870, 15 000, 35 000, 49 300, 200 000, 470 000, 803 000, 1 260 000, 2 700 000, 3 150 000, 3 390 000, 4 110 000, 6 590 000) and had a polydispersity lower than 1.10 except for the standards with a mass above 3000000, which had a polydispersity lower than 1.3.

The oligodeoxynucleotide standard [a mixture of $(dT)_{12}$ to $(dT)_{18}$] was purchased as sodium salt from Pharmacia (Uppsala, Sweden). The synthetic oligonucleotide $(dT)_{16}$ was ordered from Microsynth (Balgach, Switzerland) and used without further purification. The peptide standard was obtained from Sigma (St. Louis, MO, USA).

2.2. High-performance liquid chromatography

The Ultimate fully integrated capillary HPLC system (LC Packings, Amsterdam, The Netherlands) was used for all chromatographic measurements except the inverse size-exclusion experiments where HPLC was carried out using a micro pump (model Rheos 2000, Flux Instruments, Karlskoga, Sweden) controlled by a personal computer with Janeiro II software (Flux Instruments), a microinjector (model C4-1004, Valco Instruments, Houston, TX, USA) with a 20 nl internal sample loop, a variable-wavelength detector (model UltiMate UV detector, LC Packings), and a Personnal Computer-based data system (Chromeleon 6.00, Dionex-Softron, Germering, Germany). The primary flow rate and hence the pressure was set at the micropump, the flow was split using a tee-piece and a restriction capillary (100 cm × 50 μm i.d.), and the resulting flow rate through the column was measured at the column exit. The use of tetrahydrofuran as mobile phase necessitated the use of stainless steel tubes for all high-pressure connections. The detection cell was in all cases a 3 nl ULT-UZ-N10 cell (LC Packings).

Monolithic poly(styrene–co-divinylbenzene) (PS–DVB) capillary columns were prepared according to the published protocol [29] and have been commercialized as Monolith by LC Packings. Octadecylated PS–DVB particles (PS–DVB–C_{18}) were synthesized as published in the literature [38]. The PS–DVB–C_{18} stationary phase has been commercialized as DNASep by Transgenomic (Santa Clara, CA, USA). Granular capillary columns were prepared according to the procedure described in [34].

3. Results and discussion

3.1. Column permeability

Porous polymeric stationary phases in contact with organic solvents often lack sufficient mechanical strength and the polymer may be deformed under the pressure gradient

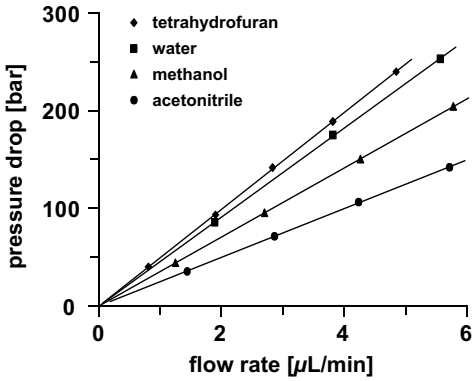

Fig. 1. Graph illustrating plots of pressure drop vs. flow velocity of different liquids. Column, PS–DVB monolith, 60 mm × 0.2 mm; mobile phases, (◆) tetrahydrofuran, (■) water, (▲) methanol, (●) acetonitrile; temperature, 20 °C.

normally encountered in HPLC columns. In order to evaluate the mechanical stability of our column material, the pressure drop across the column was measured upon perfusing it with various solvents in a wide range of flow rates. Fig. 1 shows the effect of flow rate on the back pressure in a monolithic capillary column for four different solvents. An excellent linear dependence of the column inlet pressure on the flow rate is indicated by a regression factor R better than 0.9998 for all measured curves. Thus, with any given solvent the permeability of the 60 mm × 0.2 mm i.d. column was invariant up to at least 250 bar column inlet pressure and no impairment of the column integrity occurred. This confirms that the rod is not compressed even at high flow rates.

Using water, a flow rate of 1.9 μl/min caused a back pressure of 98 bar. For methanol and acetonitrile a smaller pressure drop was registered and the order of permeability of the column agrees with that expected when comparing the viscosities of the utilized solvents (Table 1). However, the highest back pressure at equal flow rate was registered when using tetrahydrofuran as solvent (Table 1). According to theory, for a given porous structure with a given column permeability, the pressure drop through a column at a given flow rate is only dependent on the viscosity of the solvent. Therefore the back pressure of tetrahydrofuran should be between that of methanol and acetonitrile. Yet, if the pore structure and hence the permeability of the column changes depending on the utilized solvent, this relationship is no longer valid. Specifically tetrahydrofuran caused a distinctive swelling of the polymer rod in contact with the organic solvent, the permeability decreased and the backpressure was higher than initially expected. However, this observation is not really relevant for HPLC since tetrahydrofuran is hardly ever used as mobile phase component, and all other solvents (water, acetonitrile, methanol), which are common solvents for HPLC, do not cause any considerable swelling of the chromatographic bed.

Table 1
Viscosity of solvents and pressure drop per flow rate

Solvent	Viscosity η (kg/(ms))	Pressure drop/flow rate (bar/(μl/min))
Water	1.002×10^{-3}	45.6
Methanol	0.597×10^{-3}	35.4
Acetonitrile	0.360×10^{-3}	25.0
Tetrahydrofuran	0.486×10^{-3}	49.6

Finally, numerical values for the specific permeability of a monolithic capillary column were determined. Acetonitrile and water were passed through a 55 mm × 0.2 mm monolith at a pressure of 90 bar and a temperature of 20 °C. The linear flow velocity was 1.3 mm/s for acetonitrile and 0.58 mm/s for water. The specific permeability B_0 of the column was 2.9×10^{-15} m^2 for acetonitrile and 3.5×10^{-15} m^2 for water. The specific permeability with water is thus 20% higher than with acetonitrile. This indicates that some swelling of the stationary phase and restriction of the accessible pore volume occurs also with acetonitrile, yet to a much lesser degree than with tetrahydrofuran. The apparent particle diameter d_p was calculated for acetonitrile as a solvent using the Kozeny–Carman equation. A volumetric flow rate of 3.0×10^{-11} m^3 s^{-1} and an elution time of the unretained compound of 43.3 s yielded a porosity of 0.75. Using the specific permeability given above, a value of 280 nm was calculated for the apparent particle diameter.

The monolithic columns were synthesized to exhibit hydrodynamic properties comparable to that of packed columns. The back pressure in a 6 cm long monolithic column at a flow rate of 3 μl/min water was typically in the range of 90–120 bar, which compared well to a column packed with PS–DVB–C_{18} beads of equal dimensions which exhibited a back pressure of 150 bar. The lower back pressure in monoliths is an indication of an increased total column porosity.

3.2. Batch-to-batch reproducibility of column fabrication

Since the identification of individual components by HPLC is usually based on comparison of retention time, a high reproducibility of retention is a prerequisite for any chromatographic method. Retention times should show only a slight fluctuation between runs on one and the same column and between runs on different column batches, respectively. In general, the run-to-run reproducibility is an indicator for the quality of the HPLC system and is typically in the range of better than 1.0%. The batch-to-batch reproducibility, on the other hand, reflects the reproducibility of column fabrication.

In order to evaluate the batch-to-batch reproducibility of the two column types, the retention times of a mixture of seven homologous oligothymidylic acids ranging in size from 12 to 18 nucleotides eluting from 17 different 60 mm × 0.2 mm i.d. monolithic PS–DVB columns and from 10 columns of the same dimensions packed with PS–DVB–C_{18} particles were measured. The experimental

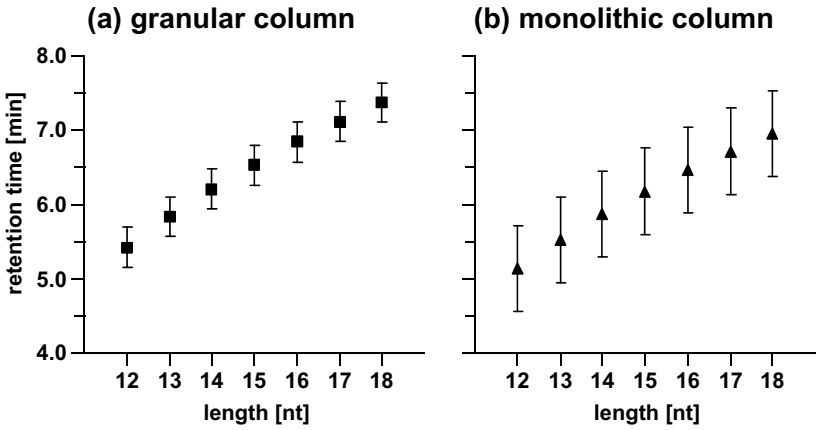

Fig. 2. Comparison of the average retention times of $(dT)_{12–18}$ on (▲) 10 monolithic and (■) 17 granular capillary columns. Columns, (▲) PS–DVB monolith, 60 mm × 0.2 mm i.d., (■) PS–DVB–C_{18}, 2.1 μm, 60 mm × 0.2 mm i.d.; mobile phase: (A) 100 mM TEAA, pH 7.0; (B) 100 mM TEAA, pH 7.0, 20% acetonitrile; linear gradient 25–60% *B* in 10.0 min; flow rate, 2.0–3.0 μl/min; temperature, 50 °C; detection, UV, 254 nm; sample, $(dT)_{12–18}$, 1.25 ng.

results are depicted in Fig. 2. The average standard deviation of the retention times among various batches of granular and monolithic capillary columns was found to be 4.2 and 9.5%, respectively, which clearly demonstrates that the uniform packing of presynthesized particles into an empty tube to form a granular column is much easier to control than the complete de novo synthesis of a chromatographic bed accomplished during the fabrication of the monoliths.

At this point it must be emphasized, that the variability in retention between different batches of monolithic columns most probably reflects slight differences in surface morphology. If the batch-to-batch reproducibility of the particle synthesis had been considered in this study, increased retention time deviations would have been observed also for the granular column format. Nevertheless, run-to-run reproducibility of retention times on a single monolithic column of 0.5–3% is well within the values that are characteristic for capillary chromatographic systems [39]. Consequently, monolithic column production requires a careful control of synthetic conditions and a rigorous selection of synthesized columns, if high reproducibility of analyte retention times between column batches is obligatory. Nevertheless, this is not a primary concern for routine proteomic and genomic applications, in which most of the important information is extracted from the mass spectral data that are not influenced by slight shifts in chromatographic retention.

Another interesting difference between the two column types can be deduced from the average retention time values (Fig. 2). On average the oligonucleotides eluted 24 s later from the granular column than from the monolithic column. Since the PS–DVB particles were octadecylated, they showed a higher hydrophobicity than the untreated PS–DVB monolithic stationary phase. Therefore a higher amount of acetonitrile was necessary to elute the components of the test mixture from the granular column than from the monolithic column.

3.3. Study of molecular diffusion processes within the chromatographic bed

Recently, we have evaluated the chromatographic efficiency of monolithic columns by isocratic elution of an oligonucleotide at 50 °C column temperature. The number of theoretical plates exceeded 11 500 plates for a 60 mm column, corresponding to 191 000 theoretical plates per meter clearly demonstrating the outstanding separation efficiency of the monolithic capillary columns [29]. Extending this communication we are presenting here the study of molecular diffusion processes within the chromatographic bed of capillary columns. For this purpose we measured the dependence of the height equivalent to a theoretical plate (HETP) from the linear flow rate by injecting the oligonucleotide $(dT)_{16}$ as test substance. The plate height curves for a typical monolithic and a typical granular capillary column are depicted in Fig. 3. Additionally, these plots were used to determine the optimum flow rate for the separation of nucleic acids by RP–HPIPC.

For the monolithic column, a minimum plate height of 8.6 μm was determined at a linear flow velocity of 0.71 mm/s, which corresponds to a volumetric flow rate of 0.97 μl/min, whereas a minimum plate height of 30.8 μm was observed for the granular column at a linear flow velocity of 0.51 mm/s, which corresponds to a volumetric flow rate of 0.59 μl/min. Obviously, using optimum flow rate conditions the monolithic column showed a 3.6 times better separation efficiency than the granular column. However it must be considered that for most applications the maximum column performance is not required. An increase in plate height by 40% on the monolithic column when using a flow rate of 2.03 instead of 0.97 μl/min is often acceptable, because the analysis time is shorter by a factor of more than 2. In fact, the total retention time of the analyte is 1.95 min at a flow rate of 2.03 μl/min and 4.23 min at a flow rate of 0.97 μl/min. But even at high flow rates the monolithic

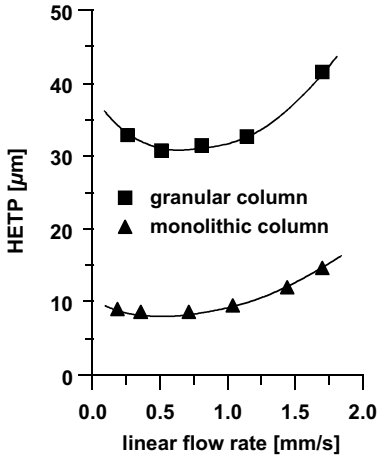

Fig. 3. Van Deemter plots for $(dT)_{16}$ characterizing (▲) a monolithic and (■) a granular capillary column. HETP values are not corrected for extra column dispersion. Columns, (▲) PS–DVB monolith, 55 mm × 0.2 mm i.d., (■) PS–DVB–C_{18}, 2.1 μm, 55 mm × 0.2 mm i.d.; mobile phase, 100 mM TEAA, pH 7.0, 8% acetonitrile; flow rate, 0.17–1.89 mm/s; temperature, 50 °C; detection, UV, 254 nm; sample, $(dT)_{16}$, 500 fmol.

column yielded a 2.5 times lower plate height compared the granular column operated at optimum flow rate conditions.

To study the differences of the molecular diffusion processes in granular and monolithic columns, we used the simplified Van Deemter equation [40] for characterizing the axial dispersion. To determine the portions of the individual band broadening processes to the overall band broadening within the chromatographic beds of the two column types, Van Deemter functions were fitted to the measured plate height curves yielding the three parameters *A*, *B*, and *C*, which characterize Eddy dispersion, longitudinal diffusion, and mass transfer, respectively. According to theory, a major difference between the mass transfer characteristics of monolithic and granular columns was expected, whereas only a little change in Eddy dispersion and no variation in longitudinal diffusion was anticipated.

The results of the curve fits, which are summarized in Table 2, showed that all three parameters were two to five times better on the monolithic column than on the granular column. Surprisingly, the mass transfer term showed the smallest improvement of all three parameters. We believe that due to the micropellicular configuration both of particles and monolithic beds, rapid mass transfer is possible with both column types and therefore, the difference in the C term was relatively small. Since the monolithic nodules were three to four times smaller than the PS–DVB–C_{18} particles (compare Fig. 3 in [29]), the improved Eddy dispersion properties of the monolithic column type was reasonable. The improvement in longitudinal diffusion has to be explained by a decrease in the labyrinth factor, in which longitudinal diffusion is hindered by the walls of the pores present in the monolithic structure.

Table 2
Parameters describing the molecular diffusion processes within a chromatographic bed

Column type	Eddy dispersion, *A* (μm)	Longitudinal diffusion, *B* (μm mm/s)	Mass transfer, *C* (μm/(mm/s))
Granular	15.7	3.6	13.5
Monolithic	3.0	0.9	6.1

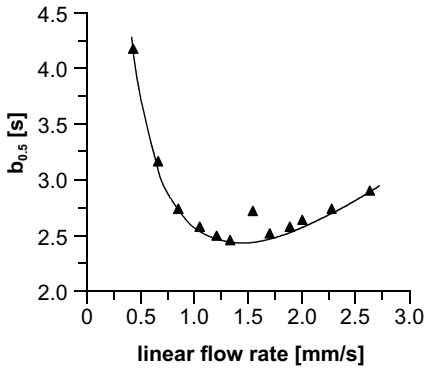

Fig. 4. Peak widths at half height of $(dT)_{16}$ eluted in gradient mode from a monolithic capillary column at different flow velocities. Column, PS–DVB monolith, 60 mm × 0.2 mm i.d. mobile phase: (A) 100 mM TEAA, pH 7.0; (B) 100 mM TEAA, pH 7.0, 20% acetonitrile; linear gradient 25–60% *B* in 10.0 min; flow rate, 0.43–2.63 μl/min; temperature, 50 °C; detection, UV, 254 nm; sample, $(dT)_{12–18}$, 1.25 ng.

At a later stage, the chromatographic performance was evaluated at different flow rates during gradient elution. A mixture of the seven oligothymidylic acids $(dT)_{12}$ to $(dT)_{18}$ was separated at various flow rates on a monolithic capillary column using a gradient from 5 to 12% acetonitrile in 100 mM TEAA in 10 min. Fig. 4 shows the dependence of the peak width at half height ($b_{0.5}$) from the volumetric flow rate for $(dT)_{16}$. A minimum $b_{0.5}$ of 2.46 s was determined at a linear flow velocity of 1.33 mm/s, which corresponds to a flow rate of 2.0 μl/min. For flow rates from 0.85 to 2.27 mm/s, the peak width showed little variation and ranged from 2.46 to 2.75 s using constant gradient conditions. Thus, in gradient elution mode the optimum in separation performance is found at a higher flow rates than in the isocratic mode. This finding is favorable since it enables the rapid, high-performance separation of biopolymers using high flow rates.

3.4. Porosity

Inverse size-exclusion chromatography (ISEC), as first reported by Halász and Martin [41], was utilized to reveal differences in porosity between different column configurations. The porosities and pore-size distributions of two monolithic capillary columns and one granular capillary column were determined by using tetrahydrofuran as solvent and polystyrene (PS) standards of different molecular mass. However, due to the fact that it is based on several assumptions, this method is not absolutely undisputed, yet presents an appropriate way to perform such measurements

as it works at least under conditions similar to those used in actual HPLC separations. Moreover, pore size distributions measured with tetrahydrofuran as solvent, which has been shown to swell the monolithic column bed (see Section 3.1), will certainly differ from the pore structure experienced by analytes chromatographed and non-swelling hydro-organic elution conditions common for protein, peptide and nucleic acid separations.

To investigate the relationship between separation performance and column porosity, a monolith of very good and a monolith of moderate separation performance were characterized by ISEC and the data obtained were compared to those measured with a particle-packed column of a performance slightly lower than that of the good monolithic column. For the evaluation of the performance of the three different columns, the average peak widths at half height $b_{0.5}$ of a mixture of $(dT)_{12}$ to $(dT)_{18}$, which was separated using a gradient from 5 to 12% acetonitrile in 100 mM TEAA in 10 min, were determined. The monolithic columns exhibited an average $b_{0.5}$ of 2.7 s and 9.7 s, respectively, while the granular column yielded a value of 3.9 s for this parameter.

From the theory of SEC we learn that the smaller a molecule the more it can penetrate into the pores of a stationary phase and the later it will elute from the column. Therefore the elution volume (V_e) of small molecules, such as toluene, represents the total volume of pores of all sizes in the rod, including the large channels. The polystyrene standards of a molecular mass between 440 and 6 590 000 gradually eluted faster, consistent with the smaller accessible volume in the column for samples of these sizes. It is important to note here that only pores with a diameter ranging from 2 to 650 nm could be probed with this approach and that the presence of pore sizes beyond this range cannot be excluded.

Using the data sets obtained from inverse size-exclusion chromatography, the cumulative porosity was calculated as the portion of V_e in the total volume of the empty tube (V_K). Fig. 5a shows a graph of the cumulative porosity against the pore size obtained for the three columns. The monolith that showed a good separation performance featured pores with a diameter as small as 5 nm. The upper exclusion limit and hence the maximum pore size was in the range of 200–300 nm. On the contrary, the monolith that exhibited poorer separation performance completely lacked pores with a diameter lower than 20 nm. The curve indicated that the average pore diameter was shifted to higher values. An upper exclusion limit could not be determined with ultimate certainty, because no polystyrene standards were available to probe the very large pores.

The particle-packed capillary column was expected to show little size-exclusion effects due to the use of non-porous PS–DVB–C_{18} particles. However, the cumulative porosity curve indicates that pores with diameters in the range between 5 and 100 nm were present. A visual inspection of the surface morphology of the monolithic and particulate stationary phases (compare Fig. 3 in [29])

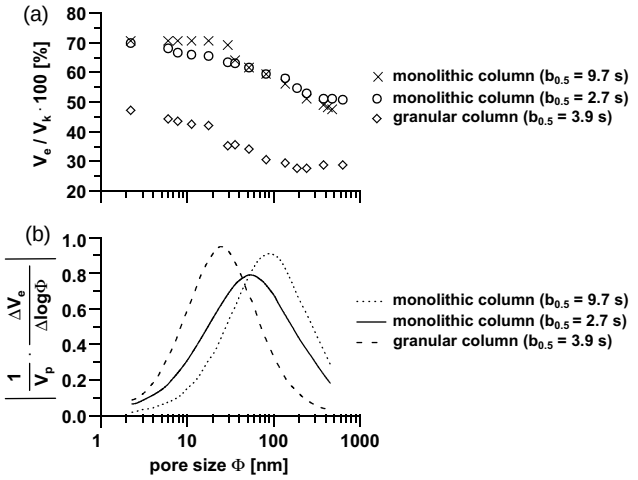

Fig. 5. Pore size distribution for (×, ○) two monolithic and (◇) one granular column determined by inverse size-exclusion chromatography. Columns, (×, ○) PS–DVB monolith, 60 mm × 0.2 mm i.d., (◇) PS–DVB–C_{18}, 2.1 μm, 60 mm × 0.2 mm i.d.; mobile phase, tetrahydrofuran; flow rate, 1.1 μl/min; temperature, 20 °C; detection, UV, 254 nm; samples, 20 nl polystyrene standards, 1 mg/ml each.

clearly revealed that the surfaces of both materials exhibited surface roughness. The observed high separation efficiency of both materials for biopolymers suggested the presence a rough surface, which, on the one hand, offer fast mass transfer properties, and which, on the other hand, increase the overall surface area and consequently the loading capacities of the columns.

The total porosity values of the examined monolithic columns were very similar and in the range of 70–71% (Table 3). This is not surprising since the ratio of monomers to porogens is identical for both monoliths tested in this analysis. However, these values exceeded the actual amount of porogenic solvent added to the polymerization mixture (60%, v/v) and reflect the contribution of volume shrinkage during polymerization. Although the packed column exhibited a much lower total porosity of only 47%, all three columns yielded nearly identical values for the internal porosity (V_p, 18–22%), which was calculated as the difference between the maximum and minimum elution volumes of the PS standards.

A plot of the relative pore size frequency versus the pore size (Fig. 5b) was used to determine the average pore size of the three stationary phases, which was 55 nm for the high-efficiency monolithic column and 89 nm for the medium-efficiency monolith. The average pore diameter calculated for the granular column was somewhat lower, namely 25 nm. Based on the measured average pore size values, the specific surface areas of the three columns were estimated. The high-efficiency monolithic column displayed a specific surface area in the range of 43 m^2/g, while for the medium-efficiency monolith a value of 32 m^2/g was calculated. The specific surface for the particle packed-column finally was 96 m^2/g. This result indicates that the specific

Table 3
Porosity, average pore diameter and specific surface determined by inverse size-exclusion chromatography for two monolithic and one granular column

Column	Porosity			Pore diameter (nm)	Specific surface (m^2/g)
	Pore[a]	Internodule[b]	Total[c]		
Monolith 1 ($b_{0.5} = 9.7$ s)	0.222	0.483	0.705	89	32
Monolith 2 ($b_{0.5} = 2.7$ s)	0.187	0.521	0.709	55	43
Granular column ($b_{0.5} = 3.9$ s)	0.185	0.285	0.470	25	96

[a] $\varepsilon_p = V_p/V_c$: V_p, pore volume, obtained from the difference in elution volume of totally excluded and totally penetrating sizing standards; V_c, volume of the empty separation column.
[b] $\varepsilon_z = V_z/V_c$: V_z, interstitial volume, elution volume of totally excluded sizing standard.
[c] $\varepsilon_T = \varepsilon_p + \varepsilon_z$.

surface area is an important but not the only factor, which determines column performance. Obviously other factors like morphology, accessibility of the surface and chemical structure have to be taken in consideration.

3.5. Loading capacity

The analytical loading capacity of a 60 mm × 0.2 mm i.d. monolithic capillary column for oligonucleotides was evaluated by injecting increasing amounts of $(dT)_{16}$ onto the column, eluting the sample with a gradient from 5 to 12% acetonitrile in 100 mM TEAA in 10 min, and measuring the peak widths at half height. The $b_{0.5}$ values were plotted against the sample load (Fig. 6). As long as no overloading of the column occurred, the peak width at half height remained constant. Overloading was indicated by a steep increase in the peak widths at half height. The maximum analytical loading capacity of this monolithic column for $(dT)_{16}$ was found to be in the region of 500 fmol (2.4 ng). Thus, it compares well with the analytical loading capacity of a packed capillary column of the same column dimensions that was determined to be in the range of 500 fmol for $(dT)_{16}$ as well [34]. Due to the higher separation efficiency of the monoliths, amounts of even ten times the analytical loading capacity could be loaded onto the monolithic columns with only a minor increase in the peak widths at half height. Moreover, such overloading still yielded peak widths in the range of the analytical capacity of granular columns. This result is very important in the case of complex sample mixtures that are commonly analyzed in proteomic or genomic applications and that tend to overload a column rather easily.

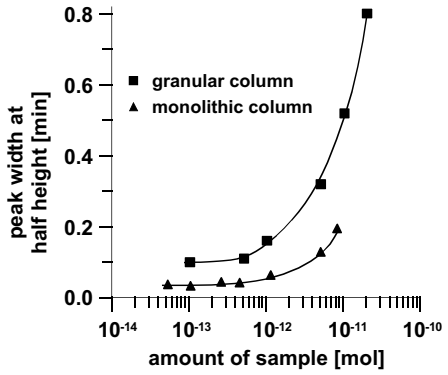

Fig. 6. Comparison of the loading capacities of (▲) a monolithic and (■) a granular capillary column (data from [34]) using $(dT)_{16}$. Columns, (▲) PS–DVB monolith, 60 mm × 0.2 mm i.d., (■) PS–DVB–C_{18}, 2.1 μm, 60 mm × 0.2 mm i.d.; mobile phase: (A) 100 mM TEAA, pH 7.0; (B) 100 mM TEAA, pH 7.0, 20% acetonitrile; linear gradient 25–60% *B* in 10.0 min; flow rate, 2.4 μl/min; temperature, 50 °C; detection, UV, 254 nm; sample, $(dT)_{16}$, 50–10 000 fmol.

3.6. Evaluation of the efficiency of monolithic columns for the separation of peptides and oligonucleotides

As described in earlier publications, monolithic PS–DVB columns were successfully employed as separation media for peptide, protein, and nucleic acid analysis [29,35–37]. In this investigation the efficiencies of 21 monolithic columns for the separation of standard oligonucleotide and peptide mixtures were investigated. For comparison of column efficiency for peptide and oligonucleotide analysis, the average $b_{0.5}$ of a mixture of $(dT)_{12}$ to $(dT)_{18}$ and of a mixture of

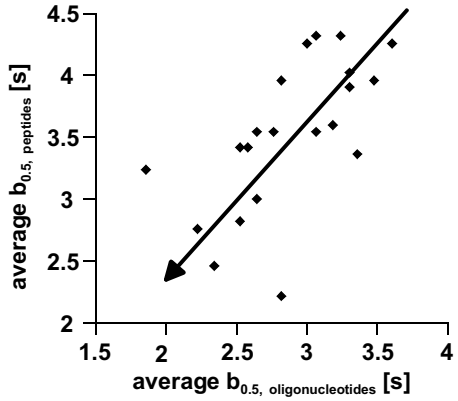

Fig. 7. Average peak widths at half height of oligonucleotides vs. the average peak widths at half height of peptides eluted from 21 monolithic capillary columns. Columns, PS–DVB monoliths, 60 mm × 0.2 mm i.d.; oligonucleotide separations: mobile phase: (A) 100 mM TEAA, pH 7.0; (B) 100 mM TEAA, pH 7.0, 20% acetonitrile; linear gradient 25–60% *B* in 10.0 min; flow rate, 2.0 μl/min; temperature, 50 °C; detection, UV, 254 nm; sample, $(dT)_{12–18}$, 1.25 ng; peptide separations: mobile phase: (a) 0.050% TFA in water; (b) 50% acetonitrile, 0.050% TFA in water; linear gradient, 0–100% *B* in 7.5 min; flow rate, 2.0 μl/min; temperature, 50 °C; detection, UV, 214 nm; sample, mixture of bradykinin fragment 1–5, vasopressin [Arg^8], [Met]-enkephalin, [Leu]-enkephalin, oxytocin, bradykinin, LHRH decapeptide, bombesin, substance P, 0.5 ng each.

nine peptides were determined. A plot correlating the average $b_{0.5}$ measured for the oligonucleotide and peptide mixtures, respectively, is shown in Fig. 7. Obviously, there is a connection between the efficiency of a monolithic column for the separation of peptides and oligonucleotides, which is indicated by the arrow in Fig. 7. However a direct prediction of the average $b_{0.5}$ of a column for one biopolymer from the value determined for the other is not possible. Most columns that were efficient for oligonucleotide separations were also efficient for peptides separations and vice versa. However, some of the columns were very efficient for one compound class while they performed poorly for the other. This suggests that the optimal column morphology necessary for a highly efficient separation of the individual types of biopolymers, namely peptides and oligonucleotides, is slightly different and that all columns need to be tested with both compounds for a complete characterization.

4. Conclusions

Monolithic columns based on PS–DVB copolymer prepared in the presence of tetrahydrofuran–decanol as porogen mixture represent highly porous separation media. This column configuration offers fast mass transfer properties, on the one hand, and high chromatographic surface area that is necessary to obtain appropriate loading capacities, on the other hand. The major advantage of the monolithic capillary columns rests within their excellent chromatographic separation efficiency for biopolymers, including peptides, proteins, and nucleic acids, which is essential for applications in proteomics and genomics.

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