Comprehensive characterization of branched polymers
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Chapter 2: Hydrodynamic chromatography of macromolecules using polymer monolithic columns

Abstract

The selectivity window of size-based separations of macromolecules was tailored by tuning the macropore size of polymer monolithic columns. Monolithic materials with pore sizes ranging between 75 nm and 1.2 μm were prepared in-situ in large I.D. columns. The dominant separation mechanism was hydrodynamic chromatography in the flow-through pores. The calibration curves for synthetic polymers matched with the elution behavior by HDC separations in packed columns with ‘analyte-to-pore’ aspect ratios (λ) up to 0.2. For large-macropore monoliths, a deviation in retention behavior was observed for small polystyrene polymers (M<sub>r</sub> < 20 kDa), which may be explained by a combined HDC-SEC mechanism for λ < 0.02. The availability of monoliths with very narrow pore sizes allowed investigation of separations at high λ values. For high-molecular weight polymers (M<sub>r</sub> > 300,000 Da) confined in narrow channels, the separation strongly depended on flow rate. Flow-rate dependent elution behavior was evaluated by calculation of Deborah numbers and confirmed to be outside the scope of classic shear deformation or slalom chromatography. Shear-induced forces acting on the periphery of coiled polymers in solution may be responsible for flow-rate dependent elution.
2.1 Introduction

Liquid chromatography (LC) is an invaluable analytical separation technique for the characterization of synthetic polymers and bio-macromolecules. Large molecules with relative molecular weights up to several millions can be separated, provided that they are well dissolved in the mobile phase [1,2]. Size-exclusion chromatography (SEC), hydrodynamic chromatography (HDC) and flow field-flow fractionation (FFF) are often used in the analysis of macromolecules. The separation conditions are typically mild (moderate temperatures and shear stress), leaving the molecules intact for further characterization (e.g. light scattering, viscometry, spectroscopy), separation, or collection of fractions. Each of these techniques separates the analytes by size in solution and enthalpic interactions between analytes and stationary surfaces must be negligible. When this is the case, the physical properties of the stationary phase, rather than the surface chemistry, are of paramount importance in creating a suitable hydrodynamic environment for separation.

As opposed to SEC, HDC separations are based on partitioning within the transient mobile phase [3,4,5]. The separation is a result of partitioning induced by surface-exclusion in flow-through pores and hydrodynamic forces on the polymer in laminar flow. Small analyte molecules can sample the low-velocity flow regions near the stationary-phase surface that cannot be sampled by larger analytes. The latter are excluded from the channel surface, because of both steric and hydrodynamic effects. An overview of conditions and requirements of separations techniques for macromolecule characterization is provided in Table 1. Hydrodynamic separations are ideally performed in very narrow open (tubular) channels, because of their well-described geometry, which allows rigorous theoretical description and calibration [6], and the absence of eddy diffusion. The selectivity in HDC depends on the aspect-ratio ($\lambda = r / R$) that relates the size of the analyte molecule (radius $r$) to the size of the flow-through channel (radius $R$). For solutes moving through open-tubular channels with laminar (Poiseuille) flow (i.e. a parabolic flow profile), the migration rate can generally be expressed as the residence time of an analyte polymer or particle ($t_p$) relative to the migration time of a small-molecule marker ($t_m$) as defined in Eq. 1 where $\tau$ is the relative retention (with $\tau = 1$ for a flow marker). In the basic form with $C = 1$ Eq.1
describes solute migration based on surface exclusion only. This is the dominant effect for low values of $\lambda$. $C$ is a variable used for including hydrodynamic effects. Its value varies between 1 and 5.3 depending on solute type and model assumptions [7].

$$\tau = \frac{t_p}{t_m} = \frac{1}{1+2\lambda - C\lambda^2}$$  \hspace{1cm} (1)

**Table 1.** Description and boundary conditions for selected size-based macromolecule separations.

<table>
<thead>
<tr>
<th></th>
<th>SEC</th>
<th>HDC</th>
<th>MTF</th>
</tr>
</thead>
<tbody>
<tr>
<td>Principal requirements</td>
<td>Stagnant pore volume.</td>
<td>Transient mobile phase + inhomogeneous flow profile (e.g. Poiseuille flow).</td>
<td>Obstructed flow for analyte molecules.</td>
</tr>
<tr>
<td>Critical dimensions</td>
<td>Stagnant-pore size related to size of analyte molecules in solution.</td>
<td>Channel diameter 5 to 50 times the diameter of analyte molecules in solution. $0.02 &lt; \lambda &lt; 0.2$</td>
<td>Channel diameter less than 2.5 times the diameter of analyte molecules in solution. $\lambda &gt; 0.4$</td>
</tr>
<tr>
<td>Implementation</td>
<td>Porous particles; monoliths with bimodal pore-size distributions.</td>
<td>Open-tubular columns ($\leq 2 \mu m$ inner diameter); packed columns (non-porous particles; $\leq 2 \mu m$ particle diameter), monoliths $\leq 1 \mu m$ channel diameter.</td>
<td>Columns packed with sub-micron (non-porous) particles; monoliths (ca. 0.1 $\mu m$ channel diameter).</td>
</tr>
<tr>
<td>Selectivity</td>
<td>Molecular size (flow-rate independent).</td>
<td>Molecular size (largely flow-rate independent).</td>
<td>Molecular size, branching (flow-rate dependent).</td>
</tr>
<tr>
<td>Stationary-phase characterization</td>
<td>Particle-size measurement (Coulter counter, SEM, FFF); MIP; Inverse SEC</td>
<td>Particle-size measurement; MIP</td>
<td>MIP, permeability</td>
</tr>
<tr>
<td>Linear (interstitial) velocity</td>
<td>0.5 mm/s</td>
<td>1 to 2 mm/s</td>
<td>0.05 mm/s</td>
</tr>
<tr>
<td>Typical column dimensions</td>
<td>$300 \times 7.5 \ mm^a$</td>
<td>$150 \times 4.6 \ mm$ (packed columns)$^b$</td>
<td>$150 \times 4.6 \ mm$</td>
</tr>
<tr>
<td>Volumetric flow rate</td>
<td>1 mL/min</td>
<td>1 mL/min (packed columns)$^b$</td>
<td>10 $\mu$L/min</td>
</tr>
<tr>
<td>Typical analysis time</td>
<td>10 min$^a$</td>
<td>4 min</td>
<td>180 min</td>
</tr>
</tbody>
</table>

$^a$ Often several columns are used in series.

$^b$ Typical dimensions of open columns for HDC would be $500 \ mm \times 1 \ \mu m$ I.D. and the flow rate would be of the order of 10 $n$L/min. Such experiments are highly impractical.
Separations of particles in open-tubular columns are extremely difficult to perform, due to the exceptionally narrow column diameters needed (internal diameter of the order of 1 µm) and the resulting brutal requirements on injection, detection and other aspects of the instrumentation [8]. HDC can more conveniently be performed on columns packed with non-porous particles. In such columns, the inter-particle space serves as a network of narrow channels where the hydrodynamic separation takes place [9]. For packed beds, the dimensions of $R$ scale with the particle size. Columns with narrow and uniformly sized flow-through channels require homogeneous packing of very small particles, which is notoriously difficult. Packing capabilities for small particles dictate the lower limit of selectivity attainable in packed-column chromatography. HDC has been demonstrated using 1-µm non-porous particles where a value of $R = 213$ nm was obtained [10]. Alternative stationary phases that provide suitable flow-through characteristics may be applied to perform HDC. As a result of advances in microfabrication, chips and pillar-structured micro channels have been used with increasing success to perform hydrodynamic separations [11,12]. However, $R$ values suitable for the separation of synthetic polymers are difficult to realize even with the most-advanced contemporary fabrication technologies.

Monolithic columns, which have become increasingly popular as separation media for LC [13], can also be considered for HDC. Hydrodynamic separations can be performed in the macropores, which offer a highly interconnected network of flow-through pores in the monolith. In contrast to the well-defined structure of packed beds with uniform particles, the structure and porous properties of monoliths may vary with the type of material and the preparation conditions. Although many different formulations and preparation techniques for monoliths have been presented in recent years [14], silica monoliths [15] and organic-polymer monoliths [16,17] have become most wide-spread in liquid chromatography. Separations of polystyrenes with low dispersity based on SEC-type partitioning have been demonstrated using silica monoliths featuring a bimodal pore-size distribution (PSD) [18]. However, the small volume of stagnant mobile phase in mesopores in comparison with the much larger external volume in the flow-through pores ($\varepsilon_i/\varepsilon_e << 1$) limits the resolution and sample capacity for SEC separations on this type of monolith. The ratio $\varepsilon_i/\varepsilon_e$ is even more unfavorable for
polymeric monoliths due to the absence of mesopores (microglobules in the polymeric material) and thus the absence of stagnant zones in the column [19].

Separations of synthetic polymers by HDC using organic-polymer monoliths have been investigated in this work. Polystyrene-co-divinylbenzene (PS-DVB) was selected as the material of the monoliths based on its mechanical strength, solvent compatibility and low susceptibility for enthalpic interactions with synthetic polymers. Due to the low degree of dimensional shrinkage during polymerization, PS-DVB columns can be prepared successfully in-situ in wide-bore stainless steel columns [20], which allows usage in a manner analogous to contemporary high-performance SEC. We will attempt to elucidate the separation mechanism by relating the observed selectivity to the morphology and the pore-size distribution.

2.2 Experimental

2.2.1 Chemicals and materials

Styrene (PS, >99.5%), divinylbenzene (DVB, ~80%), dodecanol (98%), and azodiisobutyrodinitrile (AIBN, 98%) were purchased from Sigma-Aldrich (Zwijndrecht, The Netherlands). Tetrahydrofuran (THF, 99.8% unstabilized HPLC grade), diethyl ether (99.5%), and toluene (99.7%) were obtained from Biosolve (Valkenswaard, The Netherlands). Ethanol (99.7%) was obtained from BDH Chemicals (Poole, England). 2,6-di-tert-butyl-4-methylphenol (ionol, 99%) was acquired at Acros (Geel, Belgium). Polystyrene and poly(methyl methacrylate) standards with low dispersity and relative molecular weights ($M_r$) ranging between 580 Da and 3.7 MDa were obtained from Polymer Laboratories (Church Stretton, UK).

The monomers were purified by passing them over activated basic alumina followed by a distillation under reduced pressure. AIBN was refluxed in diethylether for 30 min, re-crystallized, and dried under vacuum before use. Helium 5.0 (99,999% Praxair, Vlaardingen, The Netherlands) was used to degas the HPLC mobile phase prior to use. The polymer standards were dissolved in THF.
Stainless-steel column hardware (100 mm × 4.6 mm I.D. and 250 × 4.6 mm I.D.; SS grade 316), including end fittings, and 2-μm frits was purchased from Restek (Bellefonte, PA, USA).

2.2.2 Instrumentation

HPLC experiments were performed on a Shimadzu LC system (‘s Hertogenbosch, The Netherlands) consisting of a system-controller (SCL10a), a micro-pump (LC10Advp), a column oven (CTO7), and a UV detector (SPD10AVvp). Data acquisition was performed using ClassVP software. Separations were performed applying 5-μL injections, with the column placed in the oven thermostatted at 50°C. The flow rate was varied between 10 and 500 μL/min to record calibration curves on different monolithic materials. UV detection was performed at 260 nm or 280 nm.

Porosity data were obtained by using Pascal 140 and 440 mercury-intrusion porosimeters (CE Instruments, Milan, Italy) for low- and high-pressure analysis, respectively. The pore-size distribution was calculated using Pascal software using a model based on the Washburn equation [21] assuming cylindrical pores and a surface-contact angle of 140° for mercury with the monolith. The samples for mercury-intrusion porosimetry (MIP) were obtained by extruding the monolithic columns from their steel cladding by removing one end fitting of the column and applying a flow. The monolith was cut into coarse pieces and dried overnight under vacuum.

2.2.3 Column preparation

Monolithic columns were prepared in-situ in 4.6-mm I.D. stainless-steel columns. The composition of the polymerization mixture was 20% styrene, 20% divinylbenzene (w/w). The percentage of toluene was varied in between 10 and 24% (w/w) to control the pore size; dodecanol was used to make up to the composition (60% w/w minus the toluene content). After purging the polymerization mixture with Helium for 10 min. it was transferred into the column, closed by stainless-steel disks in lieu of porous frits. Polymerization was performed in a water bath (with Neslab RTE-140 water circulator, Thermo, Waltham, MA, USA) for 24 hours at 80°C. After completion of the polymerization reaction, the stainless-steel disks were replaced by porous frits and the columns were flushed with at least 50 column volumes of THF at 50°C and 10 μL/min.
2.3 Results and discussion

2.3.1 Preparation and characterization of monoliths for HDC

To make the polymer HDC separations compatible with conventional detectors for the characterization of macromolecules, such as refractive-index detection, viscometry, and static light-scattering, the monoliths were developed in wide-bore (4.6 mm I.D.) columns. No covalent bonding of the monolith with the wall was required since the cross-linked polymer was significantly more swollen in the SEC mobile phase (THF). For a “small” molecule (ionol) symmetric peak shapes were observed, indicating the absence of channeling effects.

To create monoliths with macropores that give inter-particle space of comparable dimensions to columns packed with sub-3 μm particles [7], the porogen ratio in the polymerization mixture was adjusted while the monomer composition was kept constant. A detailed description of pore formation and the effect of porogen composition on the phase separation and consequently on pore and globule size is provided by Eeltink et al. [22]. Figure 1 shows the intrusion curves (A) and the volume distributions (B) of the monolithic materials as determined with mercury-intrusion porosimetry (MIP). The macropore size of the monolithic materials decreased with increasing toluene content in the reaction mixture. Remarkably, the monoliths with the smallest mode pore size (< 500 nm) appear to have a bimodal pore-size distribution. This is probably an artifact of the MIP measurements, due to compression effects of the semi-flexible monoliths during the intrusion process. In the MIP experiment dried monolith (under vacuum) is immersed in mercury and subsequently pressurized. At initial conditions mercury does not protrude the pores. During the intrusion process the macropores are filled with mercury at the pressure required to overcome the surface tension of mercury to enter the pores. For the material with the largest pores (sample 1 with a macropore diameter of 1200 nm) this occurs at approximately 1.2 MPa. For monoliths with smaller pores higher pressures are required, because the intrusion pressure is inversely related to the pore size. However, these materials are compressed before the onset of pore intrusion, as shown in Fig. 1a, and this will result in an increasing bias to smaller pore size and even an apparent bimodal pore size (Fig. 1b).
Fig. 1. (a) Intrusion curves and (b) pore-size distributions of monoliths with different macropore size as determined with mercury-intrusion porosimetry. Numbers correspond to materials depicted in Table 2.
In the Appendix (section 2.5) it is discussed how extrusion data obtained by MIP may be used to confirm sample compression during intrusion measurements. Caution should be exercised in interpretation of the PSD from Fig. 1b, because this may be influenced by the extent of compression at the moment of mercury intrusion. This may result in the apparent narrow distribution, particularly for pores larger than the mode of PSD, as observed for materials 6 and 7.

Flow-restriction measurements with THF were used to compare macropore sizes for monolithic columns without errors introduced by compression of the monolith. The Hagen-Poiseuille equation (Eq. 2) can be used to relate changes in flow resistance to macropore-size, under the assumption that the monoliths have narrow pore-size distributions. It relates backpressure ($\Delta P$) and average linear mobile-phase velocity ($u_0$) in cylindrical channels to solvent viscosity ($\eta$), column length ($L$), and channel radius ($r$). This relationship has been demonstrated to hold for the pores in acrylic and styrenic monoliths [23].

$$\frac{\Delta P}{u_0} = \frac{8\eta L}{r^2}$$  \hspace{1cm} (2)

The $\Delta P/u_0$ ratio was determined for material 4, which was selected as reference for its balance between pore size and compression effects. Under the assumption that the morphology remains the same, Eq. 2 was used to convert the changes in $\Delta P/u_0$ ratio to macropore size (diameter $D_P$) for the other materials with $r = D_P/2$. Table 2 summarizes mode pore sizes as determined with mercury-intrusion porosimetry ($D_{mip}$) and flow-resistance measurements ($D_P$). The deviation between $D_{mip}$ and $D_P$ becomes larger for monoliths with smaller pores. This is indicative for compression effects in MIP.

The microscopic images obtained with scanning electron microscopy (SEM; see Fig. 2) show the typical globular structures of the monoliths prepared with different porogen composition. It was observed that monoliths with sub-micron pores have the same globular structure as their highly-permeable counterparts, but the domain size (i.e. the length scale of both pore and globular support) is different. Surface roughness of the fused globular structure for sample 1 provides some void space with dimensions significantly smaller than the through pores of 1.2 µm. Exclusion from such pores may
contribute to the separation (SEC mechanism), but because the void volume is obviously low compared to $\varepsilon$, this contribution will be small. For monoliths with sub-micron pores no large through pores were observed. Therefore, the mobile phase must be flowing through the sub-micron pores, thereby providing a suitable environment for HDC of polymers.

![Fig. 2. Scanning electron micrographs of polymer monoliths prepared with different porogen ratios. (a) material 1: 10% toluene, 50% dodecanol, 20% PS, 20% DVB, (b) material 6: 18% toluene, 42% dodecanol, 20% PS, 20% DVB.]

<table>
<thead>
<tr>
<th>Monolithic material</th>
<th>Wt% toluene in polymerization mixture</th>
<th>Mode pore size (nm) MIP $D_{mip}$</th>
<th>Mode pore size correction using Poiseuille, $D_p$</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>10</td>
<td>1170</td>
<td>1194</td>
</tr>
<tr>
<td>2</td>
<td>12</td>
<td>550</td>
<td>571</td>
</tr>
<tr>
<td>3</td>
<td>14</td>
<td>305</td>
<td>321</td>
</tr>
<tr>
<td>4</td>
<td>15</td>
<td>258</td>
<td>258*</td>
</tr>
<tr>
<td>5</td>
<td>16</td>
<td>216</td>
<td>241</td>
</tr>
<tr>
<td>6</td>
<td>18</td>
<td>127</td>
<td>162</td>
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<tr>
<td>7</td>
<td>20</td>
<td>93</td>
<td>126</td>
</tr>
<tr>
<td>8</td>
<td>22</td>
<td>50</td>
<td>104</td>
</tr>
<tr>
<td>9</td>
<td>24</td>
<td>28</td>
<td>75</td>
</tr>
</tbody>
</table>

*reference value in $D_p$ calculation
2.3.2 HDC separation of polymers

Polystyrene (PS) and polymethylmethacrylate (PMMA) standards were used to study the separation performance of PS-DVB monoliths with different pore sizes. Figure 3 shows overlaid chromatograms obtained for individual PS standards obtained on a 100 mm × 4.6 mm I.D. monolithic column with $D_p$ of 258 nm (material 4) operating at flow rates of 300 μL/min and 100 μL/min. Good peak symmetry, $A_s = b / a < 1.24$ (with $b =$ the peak width of the tail at 10% of peak height and $a =$ the peak width at the front at 10% of the peak height) was observed. The peak width at half height for 20 kDa PS was 6.1 s for the 300 μL/min separation, yielding a (minimum) plate height of 18 μm. Backpressure over the monolith was 120 bar for THF at 50°C at 300 μL/min.

Compared to the best plate-height values, about 6 μm for HDC separations reported on columns packed with 2.7-μm particles at a linear velocity of 0.5 mm/s or higher, the peaks are significantly broader [7]. Since the mass-transfer contribution to the total peak width can be neglected in HDC, peak dispersion for polymers can be attributed to the
large eddy-diffusion contribution induced by the column inhomogeneity. No significant changes in polymer separation efficiency have been observed with changes in the mobile-phase velocity (Fig. 3) or macropore size of the different monolithic materials.

Ionol is commonly used as a marker for the mobile-phase volume and its dimensionless retention was defined as $\tau = 1$. Different elution volumes were observed for other low-molecular-weight flow markers, such as benzene, toluene, and alkylbenzenes. Alkylbenzenes were found to elute earlier with increasing molecular weight, supporting a separation based on size rather than a separation based on (adsorption) interactions. Similar behaviour was observed for commercially available SEC columns with PS-DVB cross-linked porous packings (Appendix, section 2.5.2). Low-molecular-weight non-polar markers can adsorb onto or diffuse into the cross-linked PS-DVB phase. In case of the ionol both processes are unfavourable, because of its polarity. Different behavior of the flow marker may cause an offset, which should be taken into account when comparing phases with different cross-link densities or permeabilities. Monolithic columns compared in this work were all prepared with the same monomer-to-cross-linker ratio and they all behaved comparably.

High flow-rates could not be used on all monolithic materials, because of the high backpressures generated in the narrow macropores and the concomitant risk of phase compression. Separations of polystyrene standards were obtained with flow rates ranging from 300 $\mu$L/min (material 2 and 3) down to 20 $\mu$L/min for material 9. The effect of macropore size on the retention behaviour and on the selectivity window is demonstrated by the calibration curves depicted in Figure 4. Monoliths with different macropore sizes show selectivity across different molecular-weight ranges. Columns with narrower macropores (and thus lower permeabilities) separate smaller polymers. This concurs with the expectation of HDC being the dominating retention mechanism as postulated in the introduction. Selectivity for the different monolithic materials is very similar between $0.75 < \tau < 0.95$, but the corresponding molecular-weight ranges differ by more than one order of magnitude. For each monolith the effective range of separation covers at least 2 orders of magnitude in polystyrene molecular weight. For values of $\tau < 0.75$ differences in the shapes of the calibration curves were observed. For the materials with larger macropores (materials 3 through 6) the separation window
Hydrodynamic chromatography of macromolecules using polymer monolithic columns

extended down to $\tau = 0.65$, which is extraordinary for HDC-type separations. Separations at the upper end of the calibration curve have been observed to be flow-rate dependent in previous studies in which packed columns were used [7,10]. Comparison of the calibration curves in a universal format provides a better means to evaluate this hypothesis using monolithic columns. In a universal calibration graph the aspect ratio $\lambda$ is displayed on the y-axis, which allows for a direct comparison of HDC-type separations irrespective of macropore size or molecular weight of the analyte polymer.

Fig. 4. Effect of macropore size of monolithic columns on HDC selectivity for polymers with $M_r$ ranging between 990 Da and 3.7 MDa. Numbers correspond to materials depicted in Table 2. Monolith materials 2 and 3 were operated at 300 µL/min, materials 5 through 8 at 50 µL/min, and material 9 at 20 µL/min.

The size of the flow-through channel and that of the solute molecules in solution must be known to calculate $\lambda$. Neither is obvious in the case of monolithic columns and dissolved synthetic polymers. Irregular shapes of the macropores and uncertainties about the morphology prevent a straightforward calculation of the hydraulic radius (i.e. the surface-to-volume ratio), which has been successfully used to calculate the equivalent capillary size for packed beds with non-porous particles [7]. The mode of pore size from MIP is expected to be less accurate when either the pore-size distribution in the monolith is broad or when compression occurs during MIP before the mode of
pore size is reached. Therefore macropore size $D_p$ as determined from flow-restriction measurements was used in calculation of $\lambda$ (Table 2). This is appropriate, because backpressure depends on the restriction in the flow-through pores where HDC takes place by definition.

Polymers in solution do not behave as hard spheres, but as flexible chains following random coil statistics. Excluded volume of the polymer chain contributes to the coil size and varies with solvent and polymer chemistry [24]. The distance of exclusion near a surface has been used successfully in modeling retention behavior. This size is commonly referred to as the effective size and is conveniently defined relative to the radius of gyration for linear random-coil polymers [25] as

$$r_{eff} = \frac{\sqrt{\pi}}{2} r_g$$  \hspace{1cm} (3)

The relation between molecular weight ($M$) and radius of gyration ($r_g$) in THF as obtained using light scattering [26] was substituted in Eq. 3. The effective size ($r_{eff}$) of PS and PMMA polymer standards was calculated using Eq. 4 and Eq. 5.

$$r_{eff,PS} = \frac{\sqrt{\pi}}{2} 0.0118 M^{0.600}$$  \hspace{1cm} (4)

$$r_{eff,PMMA} = \frac{\sqrt{\pi}}{2} 0.0110 M^{0.596}$$  \hspace{1cm} (5)

The same calibration curves for columns with various pore sizes in Fig. 4 are presented in the form of a universal HDC calibration plot in Fig. 5a. A theoretical curve for HDC on packed columns (calculated using Eq. 1 and $C = 2.7$) is provided for reference purposes [9]. Experimental data match the theoretical curve for HDC separation best for solutes in the center of the selectivity window of the columns (around $\lambda = 0.1$). The slope in this central region is identical for all curves, which suggests that the balance between size exclusion and hydrodynamic effects is identical to that encountered with HDC in capillaries and packed beds.

The experimental curves do not coincide with the theoretical curve, with an offset towards lower elution volumes that increases with macropore size. This offset is
believed to result from additional size-exclusion effects. For \( \lambda < 0.1 \) size exclusion of the polymer from the walls of flow-through channels is the main mechanism of separation [6]. Modeled retention assumes surface exclusion in cylindrical channels. Globular morphology and a distribution of the pore sizes (PSD) of the monolith, however, provide an increased volume for SEC effects. For macropores with a large average diameter this may result in increased selectivity at low \( \lambda \). The broad PSD for materials 1 and 2 (Fig. 1B) and increased selectivity of material 2 for \( \lambda < 0.02 \) (Fig. 5a) illustrate this effect on monolithic columns. Size-exclusion effects other than wall exclusion in flow-through pores observed for \( \lambda < 0.02 \) decrease with macropore size and account for < 5% in elution volume for all monoliths. This effect is different from exclusion in HDC using columns packed with non-porous particles, which is limited to the geometric exclusion volume of spheres and scales with particle size [27]. It is dependent on the morphology of the monolith and may, therefore, be reduced further by optimization of the column-preparation process.

### 2.3.3 Flow-rate dependence in polymer separations

Flow-rate dependent elution behavior was observed for polymers separated at \( \lambda \sim 0.2 \) and above (Figs. 5 and 6). Hydrodynamic interactions (particle rotation, drag, flow-induced radial force, etc.) become significant for solutes approaching the flow-through channel size and depend on both flow rate and solute characteristics [9]. Only when these contributions hold universally, retention will scale with \( \lambda \) and a single constant can be used to account for hydrodynamic interactions in Eq. 1 (e.g. \( C = 2.7 \), assuming rotating, non-draining behaviour of polymers in cylindrical channels according to Dimarzio & Guttman [28]). However, this universality fails for \( \lambda > 0.4 \) and the selectivity becomes dependent on either macropore or polymer size (Fig. 5a). For materials 5 through 8 the calibration curve for monoliths with smaller macropores demonstrates stronger reversal due to stronger retardation by hydrodynamic effects. The same calibration curves acquired at 20 µL/min (Fig. 5b) closely resemble the theoretical curve, which predicts strong retardation at \( \lambda > 0.2 \) after the assumption of non-draining polymer coils. At \( \lambda = 0.4 \) reversal of the calibration curves towards higher \( \tau \) values is observed. Both PS and PMMA polymers are present as random coils under good-solvent conditions and their flow-rate dependent elution behaviour is identical (Fig. 6).
Fig. 5. Universal retention plot showing the calibration curves on monoliths with different macropore size. (a) Materials and LC condition similar as in Fig. 4. (b) Reversal of calibration curves for material 5 through 8 when operating at a flow rate of 20 μL/min.
Fig. 6. Flow-rate dependent calibration curves of PS and PMMA polymers on monolith material 7 (a) and corresponding chromatograms for PS standards 1.1 MDa, 523 kDa, 200 kDa, 71 kDa, 20 kDa, 7 kDa and 2 kDa at a flow rate 50 μL/min (b). Column dimensions: 250 mm × 4.6 mm I.D. monolithic column.
Flow-rate dependent elution of high-molecular weight polymers has been observed for separations under wall-exclusion (HDC-like) conditions in other studies [7,10,29,30,31]. It has been attributed to either deformation or elongation of the polymer coil. The time-averaged coil size measured perpendicular to the flow direction will decrease when the polymer molecules are subjected to shear stress. To describe these effects the Deborah number \((De)\) can be introduced [29]. \(De\) expresses the ratio of hydrodynamic (elongation) forces to Brownian (relaxation) forces. For dilute polymer migrating through packed beds it can be described as follows

\[
De = K_{deb} \frac{\bar{v}}{d_p} \frac{6.12 \Phi \eta r_g^3}{R T}
\]

where \(K_{deb}\) is a constant (with a typical value of 6 [29]), \(\bar{v}\) is the superficial solvent velocity, \(d_p\) the particle size of the packing, \(\Phi\) the Flory-Fox parameter, \(\eta\) the solvent viscosity, \(r_g\) the radius of gyration of the polymer, \(R\) the gas constant and \(T\) the absolute temperature.

Application of Eq. 6 for monoliths is complicated, because reference data only exist for packed beds [29,32]. In the elongation factor in Eq. 6 \((K_{deb} \bar{v}/d_p)\) the particle size can be replaced by the hydrolic radius (\(i.e.\) the radius of a capillary with an identical surface-to-volume ratio). For a packed bed of non-porous monodisperse particles \(R_h = 2/9 \ d_p\) assuming a porosity of 0.4 [5,7]. This relation was used successfully in comparing HDC selectivity between packed beds and capillary columns, but may be used for monoliths as well. For monoliths \(R_h = D_P / 2\) was used. \(K_{deb}\) is a constant that accounts for the effect of pore structure on elongation. It is determined semi-empirically by matching selectivity changes in HDC for well characterized systems with \(De = 0.5\) [32]. Since \(K_{deb}\) could not be established accurately for monoliths, the typical value for particle-packed beds was used.

Random spherical coils prevail at low values of \(De\). The onset of polymer deformation is commonly assumed to occur around a value of \(De\) of 0.1. At still higher values \((De > 0.5)\) the chains become completely elongated, resulting in a separation mechanism termed “slalom chromatography” to picture the migration of flexible, stretched polymer chains through the interstitial channels of the support [31]. Liu et al. describe a system
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with λ values on the order of 0.1 (dp = 15 µm, Rn = 3.3 µm, polystyrene rg = 125 to 450 nm). On the 4.6-mm I.D. column used the onset of slalom chromatography was observed for flows in excess of 0.1 mL/min. The present separations on monoliths differ significantly from those described by Liu on packed columns in terms of analyte molecular weight (De ÷ rg3) and aspect ratio (λ). Uliyanchenko et al. reported on slalom chromatography for polymers in the same molecular-weight range as used in the present study. They used contemporary HPLC conditions (dp = 1.7 µm, Rh = 0.38 µm) [30] with a flow rate of 1 mL/min on 4.6-mm I.D. columns, which corresponded to De = 0.6 for the 2.0-MDa PS.

Deborah values were calculated for separations on monoliths with different macropore sizes (see Appendix, section 2.5.3). At the point where the calibration curves in Fig. 5 show a reversal towards higher elution volumes the De values were almost always much lower than 0.1. Thus, the present observations are not akin to the slalom chromatography described elsewhere [31]. Conventionally, De numbers are calculated for channels much larger than the diameter of the polymer coil (λ < 0.2). In that case the elongation (Kdeb × v/dp) can be assumed not to depend on the coil size. In the present study we consider phenomena that occur for much higher λ values. Clearly, the straightforward calculation of De values does not suffice to describe the observations in such narrow channels, where the shear stress caused by the Poiseuille flow profile only affect the periphery of the polymer coil and rotation of the coil is largely prohibited. Very large polymers with λ ≈ 1 elute faster than the average fluid velocity (τ= 1; see Fig. 5). This suggests that the polymer coils are “reptating” [33,34] through the stationary-phase channels without significant restriction. Higher flow rates cause an increase in the migration rate, which suggests that chain segments of the reptating coil move towards the faster-moving central part of the Poiseuille flow profile (assuming that the non-draining assumption holds). It appears that they no longer possess the spherical coil geometry that prevails under equilibrium conditions at De < 0.1 in the absence of constriction. The chromatographic selectivity arising from coil-reptation-based elution is large and expected to cover the complete elution window of HDC. This is supported by calibration curves obtained at different flow rates for materials 5 through 8 (cf. λ > 0.4 range in Fig. 5). It is not expected that a fully flexible polymer such as polystyrene will uncoil at conditions of moderate constriction (λ ≈ 1), because
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of fast relaxation by Brownian motion under the conditions used here. In reptation or translocation of charged polymers and biomaterials, however, complete elongation may readily occur as a result of reduced flexibility in the polymers, highly constricted pores or conditions featuring much slower relaxation due to Brownian motion [35, 36, 37, 38].

A different mechanistic explanation is therefore desired for flow-rate sensitive polymer separation in monoliths.

A useful concept from the theory of flow-rate-dependent migration in HDC is “stress-induced diffusion” (SID) [9,39]. This concept implies that polymers in Poiseuille flow migrate away from the channel walls, driven by the lower entropy as a result of elongation and reduced orientation by shear stress in this region. Migration towards the channel center (and avoiding the elongating shear forces) leads to an increase in entropy [40]. This effect is strongest at high shear rates and for high molecular weights. The same arguments can be applied to reptating coiled polymers in confined channels. Relaxation towards a spherical coil sampling the full channel diameter (natural trend to increased entropy) will result in strong internal forces near the channel walls (induced decrease in entropy). This effect is in agreement with the results for polymers eluted from confined channels in monoliths in Fig. 5. Higher \( M_r \) polymers eluting at identical \( \lambda \) from larger macropores get stronger deformed by SID due to their longer relaxation times and the calibration curve demonstrates less reversal. The mechanism described here is also in agreement with topology based separation by MTF [41,42]. Branched polymers with identical hydrodynamic size but increased segment may exhibit stronger resistance to SID compared to linear polymers under identical conditions. The mechanism of an entropy-barrier was postulated before [41], but emphasized the role of migration through orifices as compared to SID which takes place in continuous narrow channels.
2.4 Conclusions

Monolithic columns for separations of macromolecules were successfully prepared \textit{in-situ} in wide bore (4.6-mm I.D.) stainless-steel columns. The selectivity window depended strongly on the size of the macropores tuned by the ratio of porogens. HDC is the dominant mechanism of separation, since the mesoporous volume required for SEC was too small. Also, calibration curves match with elution behavior as expected for HDC separation up to $\lambda = 0.2$. Only for large-macropore monoliths, a deviation in retention behavior is observed for small polymers ($M_r < 20$ kDa), which may be explained by a combined HDC-SEC mechanism for $\lambda < 0.02$.

Macropores with much smaller hydrolic radii relative to packed columns were obtained and therefore selectivity for lower-$M_r$ macromolecules can be obtained. Our approach allowed the preparation of monoliths with a pore size as small as 75 nm and a selectivity window in HDC corresponding to a theoretical column packing with 0.17 $\mu$m particles ($D_P = 4/9 d_p$). These monoliths have limited applicability for fast size-based separations due to their low permeability. Monoliths with 258 nm macropores yielded polymer separations in the molecular weight-range common for SEC separations. Selectivity equivalent to 0.6 $\mu$m particles was demonstrated on this material with only 120 bar for THF at 0.5 mm/s on a 100 mm column (Fig. 3). Size-based separations featuring selectivity beyond what is possible with contemporary column-packing techniques are readily obtained. The efficiency of polymers monoliths for HDC may be improved further by optimization of the column heterogeneity.

For high-molecular weight polymers ($M_r > 300,000$ Da) the separation in monoliths with confining channels strongly depended on flow rate. This situation differs from other flow-rate dependent in that the shear rate is not identical throughout mobile phase sampled by the coil. Response to the high shear rate experienced in the polymer-coil periphery was suggested to result in departure from thermodynamic equilibrium geometry and flow-rate dependent elution. This hydrodynamic-based explanation was found to be in semi-quantitative agreement with experimental results for linear polystyrene polymers.
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2.5 Appendix

In this supporting information extrusion data is provided from mercury intrusion porosimetry. It is explained how this information may be helpful to confirm compression of monolithic samples during porosimetry measurements. Separation of alkylbenzenes on cross-linked polystyrene-co-divinylbenzene monoliths and SEC-particles is presented to demonstrate the absence of adsorption effects and diffusion of small molecules into the stationary phase compared to non-porous silica columns.

The calculation of Deborah numbers is explained for polymer separations on monolithic columns. Threshold values for molecular weight and $\lambda$ are presented for the separation conditions that were used in obtaining calibration curves for monolithic columns with various macropore sizes.

2.5.1 Mercury intrusion and extrusion

Mercury intrusion data for two monoliths is presented in support of the discussion on compression of monoliths. During the intrusion measurement the pressure was increased up to 300 MPa. At this pressure porosity in pores with a diameter down to 5 nm can be measured. Porosity data for monolithic materials 7 and 8 (Table 2) was obtained during both pressure increase and decrease and is presented in Fig. S-1.

Once the pressure is decreased, mercury will be extruded from pores again driven by its surface tension. The pressure where extrusion will always be somewhat lower compared to the intrusion pressure. Compression of the material during intrusion measurement will result in a higher pressure required for mercury intrusion, because the pores become smaller when the material is compressed. If the compression is a reversible process, the sample will reassume its equilibrium dimensions once it has been intruded by the mercury under high pressure. Little or no effect of compression is expected for the extrusion pressure. The higher pressure difference between intrusion and extrusion pressure for material 8 supports the assumption that this material suffers more compression compared to material 7 at the moment of mercury intrusion.

The recovery may depend on actual pore geometry as well as the rate at which pressure was reduced. For the results presented here pressure was decreased at a faster rate
compared to the pressure increase. A study of mercury extrusion under well controlled conditions can reveal useful information with respect to sample compression during the intrusion measurement. Unfortunately, such data was not acquired for the work here, because the hypothesis of compression was formed after most of the measurements were completed.

Fig. S-1. Mercury intrusion during pressure increase and decrease
2.5.2 SEC separation of alkylbenzenes and solvents on monolith

The elution for ionol, benzene, toluene and alkylbenzenes was measured to confirm that absence of adsorption effects. Ionol, benzene, toluene, ethylbenzene, propylbenzene, butylbenzene and hexylbenzene were diluted in THF before injection at a concentration of about 1 mg/ml. Detection was performed by UV at 260 nm. All separations were performed at room temperature to minimize axial diffusion. Column dimensions were 150 × 4.6 mm I.D. in each case.

(A) Monolithic material 4, $D_p$ 258 nm, 100 µL/min THF
(B) 106 Å PLgel, $d_p$ 10 µm, 200 µL/min THF
(C) Non-porous silica, $d_p$ 1.0 µm, 100 µL/min THF

The elution order in Fig. S-2 and S-3 was, from left to right, ionol, hexylbenzene, butylbenzene, propylbenzene, ethylbenzene, toluene/benzene with the lowest peak height for benzene. In Fig. S-4 all elute at the same volume, because the samples do not diffuse into or adsorb onto the non-porous silica.

![Fig. S-2. Separation of small molecules on cross-linked PS-DVB monolith (A)](image-url)
Fig. S-3. Separation of small molecules on cross-linked PS-DVB SEC particles (B)

Fig. S-4. Separation of small molecules on non-porous silica (C)
2.5.3 Deborah numbers

The polystyrene molecular weight corresponding to a Deborah value of 0.1 was calculated using Eq. 6. This specific value of $De = 0.1$ was used, because it provides the lower limit where the effects of polymer deformation may be observed. For each monolith the flow rate that was used to obtain its calibration curve in Fig. 5a was used. Common variables used in calculating $De$ were a viscosity of 0.356 Cp for THF at 50°C, a Flory-Fox parameter of $2.5 \times 10^{23}$ mol$^{-1}$ and fictive particle size of $d_p = 4/9 \ D_P$.

The results are presented in Table S-1 and Fig. S-5. The onset of deformation is reached at increasingly lower molecular weight with smaller macropore size. However, it was not reached within the classic HDC selectivity range for separations on monolithic columns.

The mobile phase flow-rate directly impacts the expected onset of deformation. Deborah scales linear with both particle size (channel size) and average linear mobile-phase velocity $u_0$. In practice the flow rate and thus $u_0$ are a result from backpressure limitations and permeability of the column. According to Hagen-Poiseuille (Eq. 2) $u_0$ scales quadratic with increasing pore size at identical backpressure. Therefore, deformation of analytes is more commonly observed for highly permeable stationary phases with large interstitial pores. $De > 0.1$ is reached at much lower backpressure, within the range of common separation conditions.

Table S-1. Lower-limits for polymer deformation according to Deborah-number calculation, expressed in PS molecular weight and $\lambda$.

<table>
<thead>
<tr>
<th>Monolithic material</th>
<th>$D_P$ (nm)</th>
<th>Flow rate (µL/min)</th>
<th>$De = 0.1$ PS $M_r$ (MDa)</th>
<th>$De = 0.1 \ \lambda$</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1194</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
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<td>300</td>
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<td>0.22</td>
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<td>3</td>
<td>321</td>
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<td></td>
<td></td>
<td></td>
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<td>1.09</td>
</tr>
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<td>50</td>
<td>2.1</td>
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</tr>
<tr>
<td>9</td>
<td>75</td>
<td>20</td>
<td>3.0</td>
<td>2.15</td>
</tr>
</tbody>
</table>
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Fig. S-5. Calibration curves on monoliths with different macropore size with diamonds indicating De = 0.1 for conditions described in Table S-1.

References