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### Nanoparticle Analysis by Online Comprehensive Two-Dimensional Liquid Chromatography combining Hydrodynamic Chromatography and Size-Exclusion Chromatography with Intermediate Sample Transformation

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## SUPPORTING INFORMATION

### **Nanoparticle Analysis by On-line Comprehensive Two-Dimensional Liquid Chromatography combining Hydrodynamic Chromatography and Size-Exclusion Chromatography with Intermediate Sample Transformation**

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## S-1 Principles of Hydrodynamic Chromatography and Effect of Flow Rate

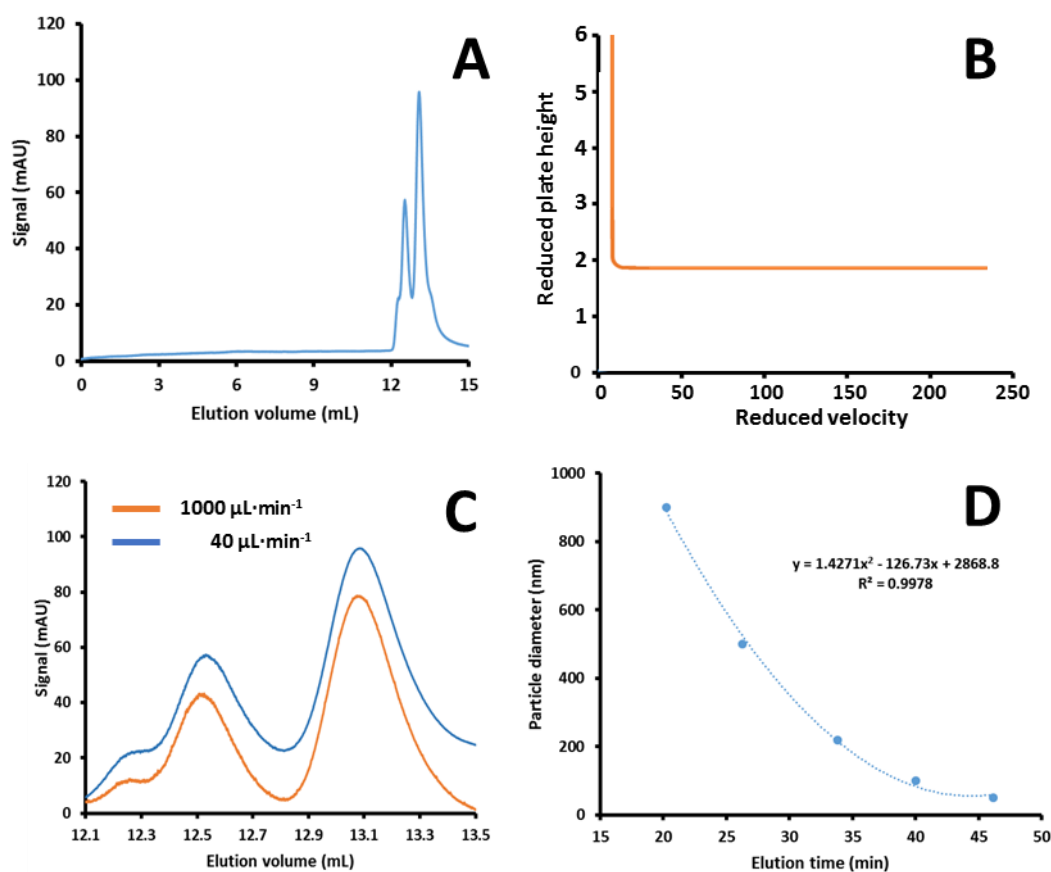
Hydrodynamic chromatography (HDC) is a technique in which macromolecules and particles are separated according to size in a column packed with solid or porous beads. It is a rapid and, in principle, convenient method to obtain a fingerprint of the size distribution of particles. The term hydrodynamic refers to the key parameter driving the separation, which arises from a non-turbulent Poiseuille flow-profile that develops in an open tube or in the interstitial medium of a packed column. For a packed column, parabolic flow-profiles develop in the through channels at Reynolds numbers less than 2000 ( $Re \approx d_p \bar{u} \rho / \eta$ , where  $d_p$  is the particle diameter,  $\bar{u}$  the average linear velocity,  $\eta$  the viscosity,  $\rho$  the density, and the units are chosen such that  $Re$  is dimensionless), such that the fastest streamline concurs with the center of the channel<sup>1</sup>.

Large particles will migrate at average velocities corresponding to the faster streamlines, whereas the smaller particles can also occupy the much slower streamlines close to the walls of the channel. The occurrence of an electrical double-layer around the particles must also be taken in account and, as is the case in size-exclusion chromatography, enthalpic interactions must be avoided<sup>2,3</sup>. The dynamic range of separation based on the HDC mechanism is determined by the diameter of the interstitial channels in packed bed columns. The aspect ratio ( $\lambda$ ) for a given HDC column is defined as the ratio of the radius of the analyte ( $r_A$ ) to that of the flow channel ( $r_C$ ). For the HDC-effect to occur,  $\lambda (= r_A/r_C)$  should be smaller than about 0.4.

The eluent for packed-column HDC typically comprises deionized water, with additives such as formaldehyde or sodium azide to prevent bacterial growth and contamination. Moreover, the material of the beads and the compositions of the eluent should be chosen such as to minimize the enthalpic interactions between the beads and the analytes (*i.e.* 'inert' beads are needed). In order to achieve this, salts and/or surfactants are added to the eluent to optimize electrostatic repulsion between the analytes and the beads of the packing<sup>4</sup>. The electrostatic repulsion determines how closely an analyte will migrate near the beads at a given ionic strength. A low ionic strength eluent corresponds to a thick electrical double layer. Assuming this layer to be stagnant, this will cause the flow channels to narrow and the HDC separation range to shift to smaller particles<sup>5</sup>.

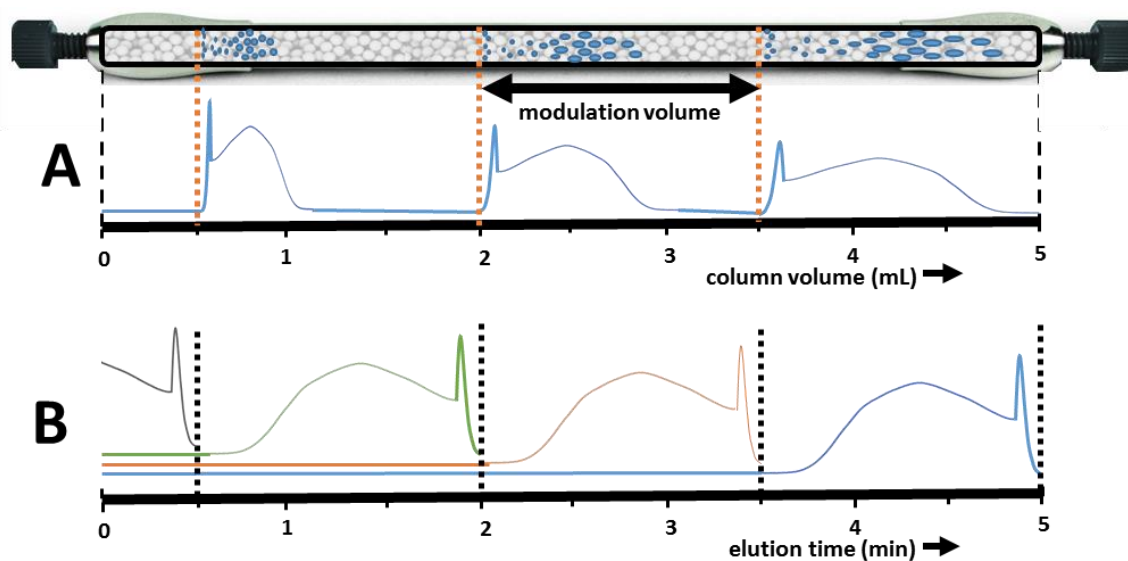
Fortunately, the band broadening does not heavily depend on the flow rate (Figure S-1B for an illustration of the theoretical van Deemter curve). This is due to that SEC is typically performed at reduced velocities higher than fifty<sup>6</sup>. For HDC of particles the reduced velocities are even much higher. This can be explained by realizing that the diffusion coefficients of such large nanoparticles are extremely low relative to small molecules<sup>2</sup>.

Consequently, we will not be punished if we temporarily increase the flow rate to flush through the first 11.9 mL of eluent and this was experimentally confirmed (Figure S-1C).



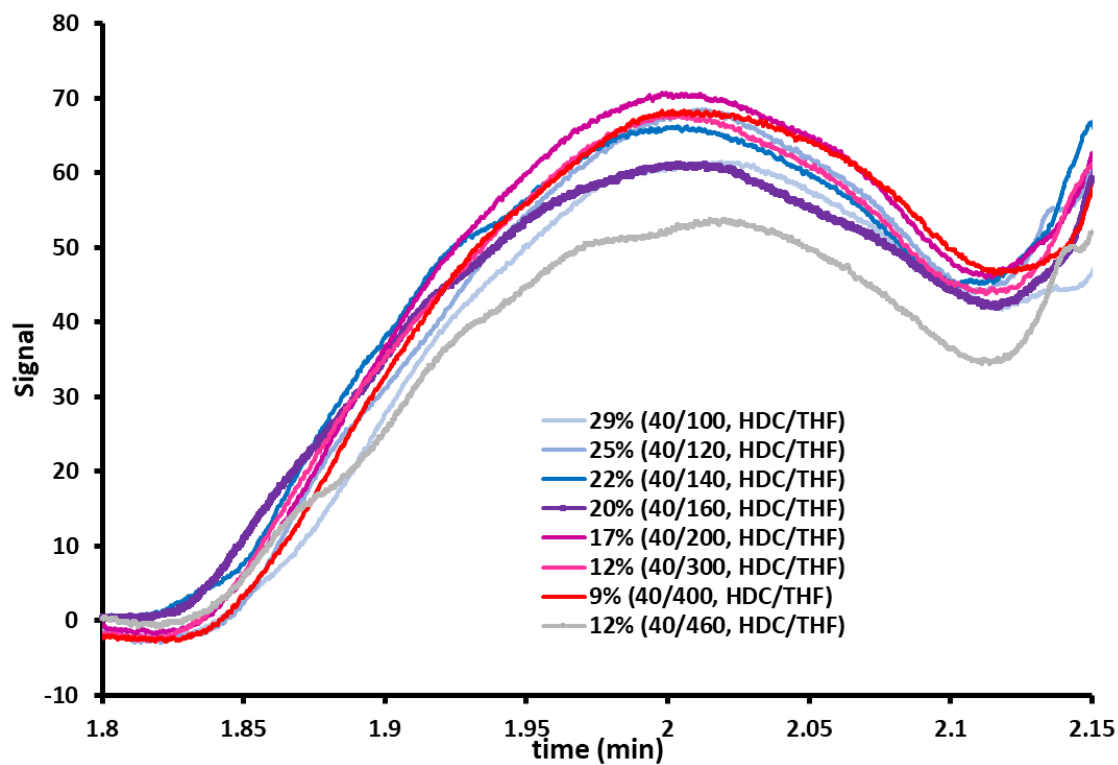
**Figure S-1:** A: Chromatogram of the separation of a mixture of PS nanoparticles (900 nm, 498 nm, 100 nm and 50 nm) by HDC using System A. B: Theoretical van Deemter plot for nanoparticles. C: Overlay of section of HDC chromatogram obtained using System A at  $1 \text{ mL}\cdot\text{min}^{-1}$  and  $40 \mu\text{L}\cdot\text{min}^{-1}$  following a flush program as described in the Analytical Methods section of the article. D: Calibration curve of the HDC separation using the flush program.

## S-2 Subsequent Injections



**Figure S-2:** Theoretical sketch illustrating the principle of overlapping injections. A: The modulation time is adapted such that the sample zones of subsequent injections do not overlap. B: The observed chromatogram after the second dimension reflects exclusively the SEC domain of the separation, provided that none of the sample components are adsorbed on the column. Note: The three SEC columns coupled together for our study are shown here as a single column for simplicity.

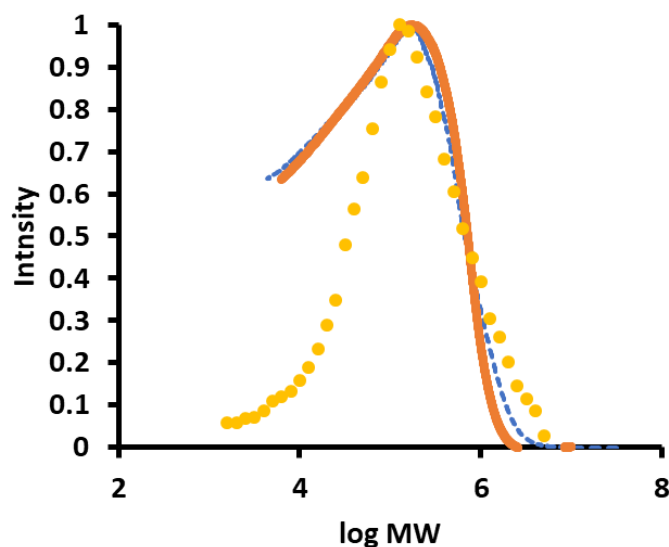
### S-3 Effect of dissolution composition on trapping yield and curve stability



**Figure S-3:** SEC chromatograms of 498-nm PS nanoparticles obtained using different solvent ratios for dissolution. All ratios are expressed in % v/v.

#### S-4 Effect of dissolution composition on trapping yield and curve stability

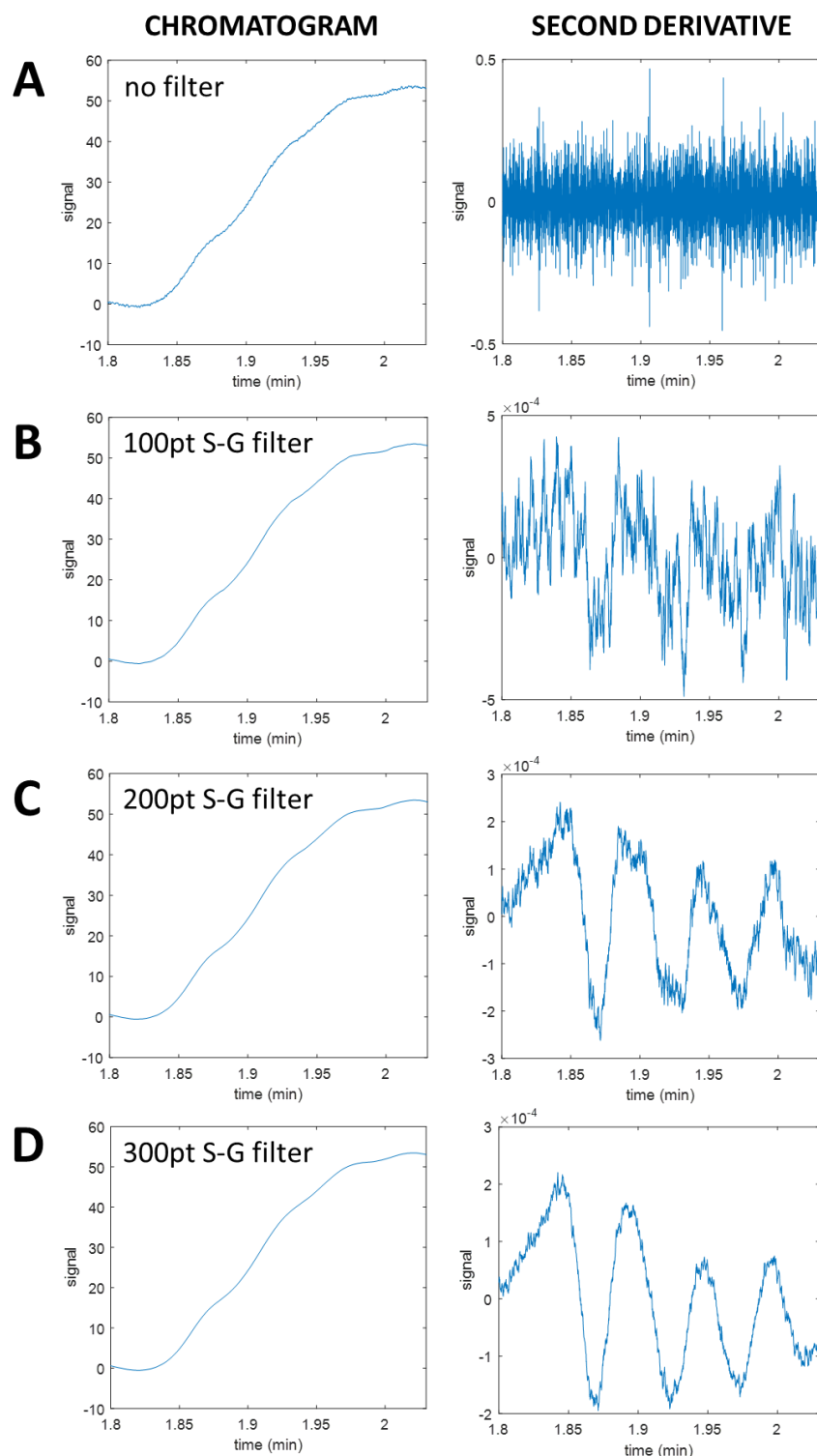
Figure S-4 shows molecular-weight distributions obtained from a single modulation from the two-dimensional HDC $\times$ SEC system, both with the first-dimension HDC column installed and using water as first-dimension eluent (solid orange line) and without the first-dimension column, using THF as first-dimension solvent (blue broken line). The high-molecular-weight side of MWDs (and the top molecular weight) are in reasonable agreement with independently obtained one-dimensional SEC data (yellow dotted line), but the low-molecular-weight side is clearly distorted. This is due to interference from the SEC baseline ( $t_0$  signal) and possibly tailing of the SEC bands in the present 2D setup. In Fig. 5 in the main text the HDC $\times$ SEC has been baseline-corrected (by subtraction of the blank signal, causing a good match between the present 2D data and off-line 1D SEC data.



**Figure S-4:** MWD of 498-nm PS nanoparticle obtained using 1D-UHPSEC (no first-dimension column installed; first-dimension eluent; blue, broken line) and 2D-HDC $\times$ SEC (first-dimension HDC separation with aqueous eluent; orange, solid line).



## S-5 Second Derivative of SEC chromatogram through a Savitsky-Golay filter



**Figure S-5:** Chromatogram signal (left) with the corresponding second derivative (right) using A) no filter, B) 101, C) 201 and D) 301 points wide Savitsky-Golay (S-G) smoothing filter. While needed to remove the disturbing background noise from the signal to allow a proper second derivative to be taken, the S-G filters do not remove the features of interest from the chromatogram.

A second-order Savitsky-Golay filter was applied with a width of 301 points, translating to approx. 2 seconds (160 Hz sampling frequency). The danger of using such a filter is – of course – (unjustly) removing relevant features of the source signal. Nevertheless, the width of the filter was necessary as can be seen in Figure 1 below.

The aim of the second derivative plot was to provide an insight to the extent of the irregularities in the polymer distribution which was expected to be continuous based on benchmark experiments in 100% THF. A second derivative plot emphasizes the inflection points present in the distribution which correspond to these irregularities.

In Figure 1A we see such irregularities in a section of the SEC chromatogram shown. However, the noise in the unfiltered signal hinders the use of a second derivative. Upon application of the S-G filter, we see the inflection points appearing as the width of the filter is increased. Moreover, the irregularities in the distribution remain present despite the level of filtering (Figure 1B, 1C, 1D) and thus – in our opinion – justify the use of this filter.

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