Journal of Chromatography A, 1532 (2018) 251–256

Contents lists available at ScienceDirect

Journal of Chromatography A

journal homepage: www.elsevier.com/locate/chroma

Short communication

Co-elution phenomena in polymer mixtures studied by asymmetric flow field-flow fractionation

Claudia Zielke[a,*], Catalina Fuentes[a,b], Lennart Piculell[c], Lars Nilsson[a]

[a] Food Technology, Engineering and Nutrition, Faculty of Engineering LTH, Lund University, PO Box 124, S-221 00, Lund, Sweden
[b] School of Chemistry, Faculty of Pure and Natural Sciences, Universidad Mayor de San Andres (UMSA), PO Box 303, Cota Cota 27 St. La Paz, Bolivia
[c] Physical Chemistry, Lund University, PO Box 124, S-221 00, Lund, Sweden

ARTICLE INFO

Article history:
Received 6 November 2017
Received in revised form
11 December 2017
Accepted 11 December 2017
Available online 13 December 2017

Keywords:
Hydrodynamic radius (r_h)
Molar mass (M) distribution
Multi-angle light scattering (MALS)
Asymmetric flow field-flow fractionation (AF4)
Glycogen
Pullulan
Poly(ethylene) oxide

ABSTRACT

Most polymers generally have complex characteristics. Analysis and understanding of these characteristics is crucial as they, for instance, influence functionality. Separation and analysis of samples of polymers, biopolymers in particular, is challenging since they often display broad distributions in size, structure and molar mass (M) and/or a tendency to form aggregates. Only few analytical techniques are suitable for the task. AF4-MALS-dRI is highly suited for the task, but the analysis can nevertheless be especially challenging for heterogeneous mixtures of polymers that exhibit wide size distributions or aggregation. For such systems, systematic and thorough method development is clearly a requirement. This is the purpose of the present work, where we approach the problem of heterogeneous polymer samples systematically by analyzing mixtures of two different polymers which are also characterized individually.

An often observed phenomenon in AF4 of samples with a high polydispersity is a downturn in M vs. elution time, especially common at high retention. This result is often dismissed as an artifact attributed to various errors in detection and data processing.

In this work, we utilize AF4-MALS-dRI to separate and analyze binary mixtures of the well-known polysaccharides pullulan and glycogen, or pullulan and poly(ethylene oxide), respectively, in solution. The results show that an observed downturn – or even an upturn – in M can be a correct result, caused by inherent properties of the analyzed polymers.

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1. Introduction

Polysaccharides are often complex in that they feature more or less wide distributions in primary structure, molar mass (M), size, conformation and/or solution behavior. Notably, many of these characteristics profoundly influence the functionality of the substances. Separation and analysis of polymers can be very challenging and few analytical techniques are suitable for the task. The challenges are typically related to the broad distributions in terms of size and M but often also to the presence of highly branched structures and/or a tendency to form supramolecular aggregates in solution. To obtain conformational and structural data over the entire size distribution of a polymer sample is therefore of fundamental importance for the understanding of their behavior. However, the separation and analysis of complex polymer samples can be very challenging, and few analytical techniques are suitable for the task.

The most commonly used analytical separation technique for polymers is size exclusion chromatography (SEC) [1]. Here, M is typically obtained either by direct determination with a multi-angle light scattering (MALS) detector or by calibration against standards. However, drawbacks with this method are its exclusion limit (upper limit of separation), which can limit the use for high M species, as well as degradation of large size species due to shear forces in the column [2], resulting in an underestimation of M [3]. Furthermore, co-elution phenomena in the presence of branched analytes may occur, as has previously been shown for polymer standards [4].

AF4 is a unique and highly versatile method that has been shown to be especially applicable to analytical separation of polymers and aggregated structures [5]. It is a chromatography-like technique based on a laminar flow of a carrier liquid along a thin separation channel, in combination with a crosswise flow, which is driving sample components to the accumulation wall at the bottom of the channel. The accumulation wall consists of a semipermeable ultrafiltration membrane through which the crossflow permeates. The induced crossflow field interacts with any macromolecule or particle in the channel and size separation occurs due to the fact that

* Corresponding author.
E-mail address: claudia.zielke@food.lth.se (C. Zielke).

https://doi.org/10.1016/j.chroma.2017.12.028
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the elution time is inversely proportional to the diffusion coefficients of the sample components and, thus, the hydrodynamic diameter. In general, sizes from approximately 2 nm up to >1 μm in diameter can be separated. For more detailed descriptions of the technique of AF4 the reader is referred to other literature [6–10]. The potential of AF4 for the characterization of polymers was demonstrated at an early stage for polysaccharides, showing fast separations with high resolution [9]. In order to obtain accurate and reliable information about sample properties, as for instance size and M, adequate detection such as multi-angle light scattering (MALS) and differential refractive index (dRI) detectors are utilized. The advantages and possibilities for analysis of polymers with AF4-MALS-dRI have been shown by several authors [11–14], also in combination with fluorescence detection for characterization of polysaccharides [15–17]. Still, M and size determination can be challenging when, for instance, analyzing samples containing ultra-large ($M > 10^7$ g/mol) branched species [18] and/or heterogeneous mixtures of biopolymers exhibiting wide size distributions. For such analytes, thorough and systematic method development is an absolute requirement.

An often observed phenomenon in AF4 is a downturn in the M vs. elution time (especially at high retention) as shown in several publications [17,19–22]. This feature is often regarded as an artifact caused by various errors in data originating from detectors and subsequent data processing, and hence not considered further. However, since the separation in the channel depends on differences in analyte hydrodynamic radii (r_h), a downturn could also be a true result, if the sample contains distinct populations of molecules that differ in their respective relations between r_h and M. Such differences could be caused by, e.g., differences in branching, mass per unit length, persistence length or solvency. Specific examples will be given below. With this work, we will show that a downturn in the M signal may indeed result from a correct analysis of a mixed polymer sample, caused by such differences in the inherent properties of the individual polymers.

In order to study the above mentioned phenomenon, we use a straightforward and simple approach, namely, to study mixtures of polymer species with suitably different properties. To this end, we have chosen the common and well-studied (in terms of size, structure and conformation) polymers glycogen, pullulan, and poly(ethylene) oxide (PEO), which are here separated and analyzed individually and as binary mixtures. Both glycogen and pullulan are polysaccharides consisting of glucose residues. However, glycogen forms hyper-branched structures with branches connected by $\alpha(1 \rightarrow 6)$ glycosidic to linear chains linked via $\alpha(1 \rightarrow 4)$ glycosidic bonds. In contrast, pullulan is a linear polymer where consecutive maltotriose units (3 glucose residues linked via $\alpha(1 \rightarrow 4)$ glycosidic bonds) are connected to each other by $\alpha(1 \rightarrow 6)$ glycosidic bonds. PEO is also a linear polymer, with ethylene oxide (—O—CH2—CH2—) as its simple repeating unit, giving it a low M per unit length. The specific polymer samples chosen in this study have overlapping M distributions, but the three different polymer types display large differences in their size (r_h) at a given M. Several publications have already shown the possibility of separating these polymers utilizing AF4 and suitable detectors [11,23–25] and pullulan is often used as a reference standard [26].

2. Experimental section

2.1. Materials and sample preparations for AF4

Glycogen from bovine liver type IX was purchased from Sigma Aldrich, Darmstadt, Germany. Pullulan with a wide size distribution (10^5 g/mol–10^7 g/mol) was obtained from Guangzhou Medcan Pharmatech LTD., Guangdong, China. Poly(ethylene) oxide PEO with a narrow molecular weight distribution (M_w = 106 000 g/mol, M_n = 101 000 g/mol given by the manufacturer) was purchased from PSS Polymer Standards Service GmbH, Mainz, Germany. The samples used for the analysis were prepared as 1 mg/mL solutions where the powder was dissolved in AF4 carrier liquid (10 mM $NaNO_3$ (Merck, Darmstadt, Germany) and 0.02% (w/v) NaN_3 (BDH, Poole, UK) dissolved in pure water from a Milli-Q system (Millipore Corp., Billerica, Massachusetts, USA)) under vigorous stirring (resulting in a pH of 7)). The injected volume per sample was 50 μl, corresponding to a polymer mass of 50 μg. The mixtures were prepared at a volume ratio of 1:1 (25 μl each).

2.2. AF4 analysis and data processing

For all experiments described in this publication, the asymmetric flow field-flow fractionation (AF4) instrument used was an Eclipse 3+Separation System (Wyatt Technology Europe, Dernbach, Germany). The system was connected to an Optilab T-rEX differential refractive index (dRI) detector and a Dawn Heleos II multi-angle light scattering (MALS) detector (both Wyatt Technology), both operating at a wavelength of 658 nm. An Agilent 1100 series isocratic pump with an in-line vacuum degasser delivered the carrier flow through the system and an Agilent 1100 series autosampler (both Agilent Technologies, Waldbronn, Germany) handled the sample injection onto the AF4 channel. A filter-holder with a 100 nm pore-size polyvinylidene fluoride membrane (Millipore Corp., uncharged) was placed between the pump and the channel inlet to ensure that only particle free carrier liquid entered the system. The separation channel used was a Wyatt long channel with a tip-to-tip length of 27.5 cm and the ultra-filtration membrane to form the accumulation wall was regenerated cellulose with a nominal 10 kDa cut-off (Microdyn-Nadir GmbH, Wiesbaden, Germany) and a 350 μm spacer. The actual thickness of the channel was 275 μm (calibration with ferritin [27]). Flow conditions were as follows: injection flow of 0.2 mL/min for 2 min (in focusing mode)+2 min of focusing without injection, channel flow Q_{out} =1 mL/min, initial crossflow Q_c = 1.5 mL/min with a linearly decreasing rate of 0.117 mL/min^2 for 12 min was applied, minimum Q_c = 0.1 mL/min, whereas the minimum Q_c was kept constant for another 10 min, the void time (t°) was calculated to be 0.6 min whereas the time before elution was 6 min. Processing of the data obtained from MALS and dRI detectors after the AF4 separation was done using Astra software in version 5.3.4.14 (Wyatt Technology Europe). M and r_{rms} were calculated using the Berry method [28,29] performing a 1 order fit with the data obtained from the chosen scattering angles 8–17 (60.0°–152.5°). For simplification, the refractive index increment dn/dc for the polysaccharides used was set to an average value of 0.14 mL/g (pullulan –0.14–0.16 mL/g, PEO –0.13 mL/g, glycogen –0.146 mL/g) [30], while the second virial coefficient A_2 was considered negligible.

3. Results and discussion

AF4-MALS-dRI results from the analysis of pullulan and glycogen individually and in mixture can be seen in Fig. 1. Fig. 1A displays the intensity of the MALS signal (left axis) of pullulan (blue), glycogen (red) and a mixture of both (black) plotted vs. the retention time (t_r) as well as their M distributions (dotted curves, right axis). Glycogen shows a peak maximum in MALS signal at t_r = 12.5 min, and a M range extending from approximately 3×10^5 g/mol to 10^7 g/mol. Pullulan elutes in a broad peak with a maximum at t_r = 18 min and a M range from approximately 10^5 g/mol to 10^7 g/mol. The two polysaccharide samples have an overlapping M range but glycogen elutes at shorter t_r at a given M which is an expected consequence of its more compact branched structure. Furthermore, striking dif-

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