Using High Speed/High Resolution Size Exclusion Chromatography Separation of Polymeric Materials with Dynamic and Multi-Angle Light Scattering Detection

Jennifer Gough¹, Robert Birdsall¹, Damian Morrison¹, John Champagne², Kyle Rogers²
¹Waters Corporation, Milford, MA USA, ²Wyatt Technology Corporation, 6330 Hollister Ave.; Santa Barbara, CA USA

OVERVIEW

- Explore more detection with the Waters® ACQUITY® Advanced Polymer Chromatography® (APC™) System and the Wyatt µDAWN™ multi-angle and quasi-elastic light scattering (MALS/QELS).
- Bring the high resolution separation capabilities of APC to light scattering analysis.
- Experience the simplicity of the APC/µDAWN hyphenation and the benefits that are brought to polymer characterization.

INTRODUCTION

Polymeric materials are an integral part of everyday tasks, and there is great complexity behind the research and manufacturing of these high quality and competitive products. From the simple task of carrying food in biodegradable shopping bags to battling diseases with intricate drug delivery systems, these everyday tasks are possible through an exacting polymer process. As technologies advance, there is a drive to understand and predict material properties through more expansive characterization and flexible analytical instrumentation. Creative analytical solutions, ranging from high speed chromatographic separation to advanced mass spectrometry, have become the answer for complex product knowledge.¹ ² ³

Traditional analytical solutions have often been the answer for polymer characterization. Coupling liquid chromatography (LC) with detectors such as a refractive index (RI), ultra violet or photo diode array (UV or PDA), evaporative light scattering (ELS) and fluorescence (FLR) have met traditional needs. Examples of creative analytical solutions include adding a secondary LC system for two dimensional (2D) chromatography or hyphenating the LC system with detectors such as dynamic light scattering (DLS) and multi-angle light scattering (MALS).⁴

LC separations with resolution and speed have not always been synonymous. Yet, with the innovative design of the Waters Advanced Polymer Chromatography (APC) system, including the robust separation column and low dispersion flow path, the system yields high resolution, short analysis times, and quick mobile phase changes. This high speed/high resolution separation technique works best with low dispersion detectors, availability and diversity of which continue to increase as the advantages of APC become more widely used in research and industry.⁵ ⁶ ⁷

In this study, the expansion of the Waters APC approach to the size exclusion separation is presented. The sample is diverted from the ACQUITY APC system to a Wyatt µDAWN with MALS/QELS and RI detector while maintaining the required system dispersion control needed for the APC experiment (Figure 1).⁸

Figure 1.
ACQUITY APC System with post column flow to the µDAWN.
EXPERIMENTAL

Conditions for demonstration measurements

The test conditions are based on using a high speed, high resolution ACQUITY APC SEC System coupled to Wyatt μDAWN and Optilab UT-rEX detectors. The two systems are connected with seven thouands inner diameter tubing and a low pressure poly fitting from the APC PDA outlet to the μDAWN inlet. The first experimental samples evaluated are a mixture of narrow polystyrene (PS) standards and of a 200K dalton PS standard dissolved in terahydrofuran (THF). The second experimental samples are a bovine serum albumin (BSA) standard and a protein standard mixture in aqueous buffer solution. The separations are analyzed using the Wyatt μDAWN and RI. Each system’s respective software is used for their respective instrument control, data collection and data processing.

SEC conditions

Organic Solvent Method

System: Waters APC System
Eluent: THF
Flow Rate: 0.7 mL/min
Sample Concentration: 1 mg/mL of each narrow standard (2 mg/mL total)
Injection Volume: 10 μL
Column Temp.: 40 °C
Columns: Waters 4.6 x 150 mm APC XT 450 Å, 125 Å and 45 Å in series
Conc. Detector: Waters ACQUITY RI detector @ 40 °C (used only when disconnected from μDAWN)
LS and RI Detector: Wyatt μDAWN and Optilab UT-rEX (settings below)
Coupling Tubing: 0.007 inches ID Stainless Steel tubing

Aqueous Method

System: Waters APC System
Eluent: Aqueous Buffer: 150mM NaCl, 20mM Phosphate, pH=6.5
Flow Rate: 0.6 mL/min
Sample Concentration: 1 mg/mL of each narrow standard
Injection Volume: 2 μL
Column Temp.: 25 °C
Columns: Waters ACQUITY BEH SEC Protein 4.6 x 150 mm 200 Å
Conc. Detector: Waters ACQUITY RI detector @ 40 °C (used only when disconnected from μDAWN)
LS and RI Detector: Wyatt μDAWN and Optilab UT-rEX (settings below)
Coupling Tubing: 0.007 inches ID Stainless Steel tubing

Wyatt Detector Conditions

LS Detector: Wyatt μDAWN
LS Temp: Ambient
RI Detector: Optilab UT-rEX (25°C)

Chromatographic System Control

Waters Empower 3 FR3 Software and Wyatt ASTRA Software
RESULTS AND DISCUSSION

The polymer samples for this study are chosen to define the separation of a polymer standard mixture and a single narrow polymer standard. The protein standards are chosen for their common use in biological and pharmaceutical fields. Comparison of the RI detector signals of the mixed polymer standards are used to assess RI resolution and analysis quality throughout the analyte flow path. The single protein is an excellent example of a biological macromolecule for confirmation of detector resolution, and the protein standard mixture adds confidence to instrumental analysis flexibility from organic solvent to aqueous chromatography within an eight hour day.

Below in Figure 2 is a chromatogram of a PS narrow standard mixture of four molecular weights. The overlaid RI analysis reveals a consistent retention time for the APC and Optilab detectors. The RI data is generated from the Wyatt ASTRA data. Data confidence is increased through the comparable instrumental signal analysis in the RI chromatogram.

Figure 3 shows the 200K Dalton (Da) PS correlation function and control curve from the µDAWN, which provides the hydrodynamic radius value. The MALS/QELS detection, in conjunction with RI detector, enable many calculations such as the absolute molecular weight, calculated mass, and mass fraction (Table 1). The constant RI detector response is indicative of peak/sample homogeneity (Figure 4). These polymer properties reveal material characteristics that can be helpful in determining behavior of the polymer in a product matrix or solution.9-11

Table 1.
Molecular weight customizable table of 200K Da PS.

![Figure 2. APC RI signal in blue, and Optilab RI signal in red of PS standard mix.](image)

![Figure 3. QELS correlation and control graphs: 200K Da PS.](image)

![Figure 4. RI is constant across 200K Da PS LS peak: consistent signal across peak.](image)
The advantages gained by polymer characterization with the µDAWN and Optilab can also be applied to the polymer-like characteristics of proteins. The BSA analysis is demonstrated below in the table and in the chromatogram, bringing confirmation to the resolution through the exhibited monomer, dimer and trimer (Table 2 & Figure 5). The hydrodynamic radius of the BSA in Figure 6 can be used to predict the behavior of the analyte in solution. The protein standard mixture, seen in Figure 7, demonstrates the separation and detection capability of the combined systems and their flexibility to operate with an organic solvent method (normal phase) or an aqueous method (reverse phase).¹⁰,¹¹

The red horizontal lines near each peak in the chromatograms below represent the molar mass distribution, and these can have linear, convex or concave shape. In this study, the BSA and protein mix exhibited some non-linear behavior in their molar mass distribution. This curvature is related to detector response of an analyte. A constant detector response is indicative of a homogenous sample that is baseline resolved from neighboring peaks in the chromatogram. Deviation from a constant detector response or non-linear response can be a result of increased peak heterogeneity or non-resolved peaks.¹²

**Table 2.**
The table is the Wyatt ASTRA calculations for the BSA molecular weight of peaks 1, 2 and 3.

<table>
<thead>
<tr>
<th>Peak 1</th>
<th>Peak 2</th>
<th>Peak 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>BSA(002)[Aqueous Demo Sequence 3]</td>
<td>BSA(002)[Aqueous Demo Sequence 3]</td>
<td>BSA(002)[Aqueous Demo Sequence 3]</td>
</tr>
<tr>
<td>Mw (kDa)</td>
<td>Calculated mass (µg)</td>
<td>Mass fraction (%)</td>
</tr>
<tr>
<td>64.794 ± 0.403 kDa</td>
<td>1.25</td>
<td>87.4</td>
</tr>
</tbody>
</table>

Average | Standard deviation | % Standard deviation | Minimum | Maximum |
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>64.794 ± 0.403 kDa</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
</tr>
</tbody>
</table>

The protein standard mixture, seen in Figure 7, demonstrates the separation and detection capability of the combined systems and their flexibility to operate with an organic solvent method (normal phase) or an aqueous method (reverse phase).¹⁰,¹¹

**Figure 5.**
LS and RI chromatogram of BSA: horizontal lines

**Figure 6.**
QELS correlation and control graphs: BSA.

**Figure 7.**
LS and RI chromatogram of protein standard mix: horizontal lines denote molar mass distribution.
CONCLUSIONS

- Coupling the APC, high resolution / high speed SEC system to the Wyatt μDAWN and RI detectors is easily managed with the selection of the correct tubing diameter.
- The impact of the Wyatt detectors on chromatographic band spread is minimized with their small inter-detector dispersion design capable of interfacing with Waters ACQUITY ultra high performance systems.
- In the example studied, the benefit of adding the MALS/QELS and RI to the SEC experiment offers the ability to calculate absolute molecular weight and hydrodynamic radius. These calculations can predict polymer behaviors in solutions/product matrices and bring confidence to sample quality through extended quantification.\(^\text{9-11}\)
- The high speed chromatography offered by the APC is matched well to the experimental conditions available with the μDAWN enabling greater characterization for the complex properties of polymers and proteins.

References