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ORIGINAL

Detection Orthogonality in Macromolecular Separations. 2: Exploring Wavelength Orthogonality and Spectroscopic Invisibility Using SEC/DRI/UV/FL

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Abstract
We continue herein the exploration of detector orthogonality in size-based macromolecular separations. Previously [5], the sensitivity of viscometric detection was juxtaposed to that of differential refractometry (DRI) and light scattering (LS, both static and dynamic), and it was shown that viscometry is a truly orthogonal detection method to both DRI and LS. Here, via the size-exclusion chromatography (SEC) analysis of blends of polystyrene and poly(methyl methacrylate), we demonstrate the orthogonality of DRI to UV detection and, within the UV region of the electromagnetic spectrum, we also explore the phenomenon of "wavelength orthogonality:" Analytes observable by one detection method are shown to be spectroscopically invisible to another method, or even to the same detection method when operating at a different wavelength. While generally focusing on blends of analytes of different molar masses (different sizes in solution), we also investigate the less-explored case of blends of coeluting analytes (same sizes in solution) where detector orthogonality can inform one's knowledge of whether or not coelution has occurred. Finally, by incorporating a fluorescence (FL) detector into the experimental set-up, we demonstrate not only its orthogonality to DRI detection but also its sensitivity to the presence of even minor (≈ 1%) fluorescent components in a sample. We hope the present experiments assist in understanding the complementarity of different spectroscopic detection methods and also help highlight the potential role of FL detection, a method which has been largely overlooked in macromolecular separation science.

Keywords Size-exclusion chromatography · Detection orthogonality · Wavelength orthogonality · Spectroscopic invisibility · Fluorescence · Macromolecular separations

Introduction

Concomitant with the ever-increasing production and use of complex polymers and blends is the need for characterization methods able to map the physicochemical phase-space occupied by these materials. Deconvoluting from each other the various chemical components present in a complex polymer or blend, or deconvoluting the chemical and physical properties of the individual components, is *de rigueur* for a proper understanding of materials synthesis, processing, and application. To this end, great strides have been made, and continue to be made, through the application of macromolecular two-dimensional liquid chromatography (2D-LC) methods to these types of samples, where the orthogonality amongst separation dimensions is meant to reflect the difference in the material properties of interest [1, 2]. Drawbacks to these methods are long analysis times and a general lack of first-principles knowledge regarding how to design interactive macromolecular LC separations.

Another way of gaining insight into the properties of complex materials comes from the application of multi-detector macromolecular LC methods, by taking advantage of the mutual complementarity of different detection techniques [3, 4]. While less attention has been paid to detector orthogonality than to separations orthogonality, the former can not only complement the latter in 2D-LC scenarios but can also, in one-dimensional separations, provide certain

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advantages over its 2D counterpart with respect to reduced analysis time and the obviating of interactive LC method design. In a previous paper [5], the detection orthogonality of the on-line viscometer vis-à-vis light scattering (both static and dynamic) and refractometric detectors was explored. The fact that the viscometer response is based on hydrodynamic transport properties of a material, whereas the light scattering detectors and refractometer responses are spectroscopically based, allowed for the former to detect samples invisible to the latter two types of detectors when experiments were conducted at conditions of so-called "spectroscopic invisibility," i.e., when sample solutions displayed virtually zero optical contrast with the neat solvent at given conditions of solvent, temperature, and wavelength of incident radiation [5].

Herein, we explore different types of detector orthogonality, namely those due to different spectroscopic processes and those due to wavelength orthogonality within a given spectroscopic process. To that end, we employ size-exclusion chromatography (SEC) with on-line ultraviolet absorption (UV), differential refractometry (DRI), and fluorescence (FL) detection to study blends of polystyrene (PS) and poly(methyl methacrylate) (PMMA) and blends of PS with and without an anthracene center-group. Additionally, for the PS/PMMA blends, we study blends of polymers of vastly different sizes in solutions, as well as the rarely explored case of blends of polymers of virtually the same size, i.e., blends of polymers which, in the latter case, coelute in a size-based separation such as SEC, and which would also coelute in a hydrodynamic chromatography or flow field–flow fractionation experiment [6–8]. These experiments are meant to further highlight the advantages of the multi-detector approach to macromolecular LC separations, in particular how taking advantage of the sensitivities of individual detection methods can better inform our knowledge of the composition of complex polymers and blends. This study also serves to showcase the usefulness of fluorescence detection; considered by many a niche method, it is, in our opinion, underutilized yet extremely powerful with respect to both sensitivity and selectivity [9–16].

Experimental

Materials

Narrow dispersity, linear PS and PMMA, designated by their peak-average molar mass (M_p) as PS 111 K ($M_p = 1.11 \times 10^5$ g mol^{-1}), PS 3.5 M ($M_p = 3.5 \times 10^6$ g mol^{-1}), and PMMA 107 K ($M_p = 1.07 \times 10^5$ g mol^{-1}), were from Agilent/Polymer Laboratories (Amherst, MA). PMMA 838 K ($M_w = 8.38 \times 10^5$ g mol^{-1}, where M_w is the weight-average molar mass) was from Scientific Polymer Products (Ontario, NY). PS center-labelled with anthracene and of $M_w = 1.08 \times 10^5$ g mol^{-1} (Polymer Source, Dorval, Canada) is referred to herein at PS-Anth-PS 108 K. In all cases, molar mass dispersity $Đ \leq 1.1$. HPLC-grade tetrahydrofuran (THF, unstabilized) was purchased from EMD (Gibbstown, NJ). All materials were used as received, without further purification.

Commercial products are identified to specify adequately the experimental procedure. Such identification does not imply endorsement or recommendation by the National Institute of Standards and Technology, nor does it imply that the materials identified are necessarily the best available for the purpose.

SEC/DRI/UV/FL Analysis

Size-exclusion chromatography analyses were performed using an Ultimate 3000 Dionex HPLC system (Thermo Scientific, Sunnyvale, CA) equipped with the following components: pump, photodiode array (PDA) detector, fluorescence (FL) detector, and on-line degasser. An Optilab T-rEX differential refractometer (Wyatt Technology Corp., Santa Barbara, CA) was used non-sequentially to the PDA and FL detectors. The instrument was computer-controlled using commercial software (Chromeleon version 6.8, Thermo Scientific). Separations were carried out on a PLgel 10-µm particle size Mixed-B SEC column (Agilent/Polymer Laboratories). The column temperature was held at 25 °C. Unstabilized THF was employed as both solvent and mobile phase, the latter at a flow rate of 1.0 mL min^{-1}. Solution concentrations were 1.0 mg mL^{-1} in THF in all cases, except for PS 3.5 M for which the concentration was 0.5 mg mL^{-1}; injection volume in all cases was 100 µL. Solutions were mixed by simple inversion and allowed to solvate, in the dark at room temperature, at least overnight.

3DFIELD chromatograms were collected using the PDA detector equipped with a deuterium lamp (190–670 nm) and a tungsten lamp (345–900 nm). In this detector, the sample is excited from both light sources in a 13 µL flow cell with a path length of 10 mm. An optical grating (490 mm^{-1}) with an entrance slit of 5 nm diffracts light to a photodiode array consisting of 1024 photosensitive elements. The PDA detector has an accuracy of ± 1 nm and reproducibility of ± 0.10 nm.

Fluorescence chromatograms and spectra were collected using a Xe flash lamp with broadband illumination from 200 nm to 880 nm. The excitation and emission monochromators have spectral bandwidth of 20 nm, accuracy of ± 2 nm, and reproducibility of ± 0.2 nm. A programmable cutoff filter wheel with five different wavelengths was used prior to detection with a photomultiplier tube. For determination of the optimal FL excitation and emission wavelengths (λ_{exc} and λ_{em}, respectively) of PS-Anth-PS 108 K,

spectra were recorded using a 30 s stop-flow function on the SEC instrument at the apex of the FL chromatographic peak.

Results and Discussion

Wavelength Orthogonality as Detection Orthogonality: SEC/UV of a PS/PMMA blend

The 3DFIELD chromatogram shown in Fig. 1a was obtained using a PDA detector from an ≈1:1 blend of PMMA 838 K and PS 111 K. PS111 K has a strong UV absorption wavelength of 260 nm while, at this same wavelength, PMMA does not absorb. This is consistent with previously reported results [17, 18]. It has also been reported that ≈230 nm is a good wavelength at which to observe PMMA [19, 20]. Shown in Fig. 1b are the UV spectra obtained from the 3DFIELD chromatogram for PMMA 838 K (Fig. 1b, top) and PS 111 K (Fig. 1b, bottom). As can be seen, 260 nm provides a wavelength for the observation of PS without interference by PMMA. At first perusal, it might appear that 235 nm provides a wavelength for the observation of PMMA without interference by PS. It is important to note, however, the different ordinate scales in the top and bottom spectra of Fig. 1b: At 235 nm, the absorbance of PMMA 838 K is 67 mAU while that of PS 111 K is 84 mAU. While 235 nm provides a minimum in the UV spectrum of PS, PS still absorbs more strongly (has a larger molar absorptivity) than does PMMA. Both polymers display near-maxima in their UV spectra at ≈217 nm.

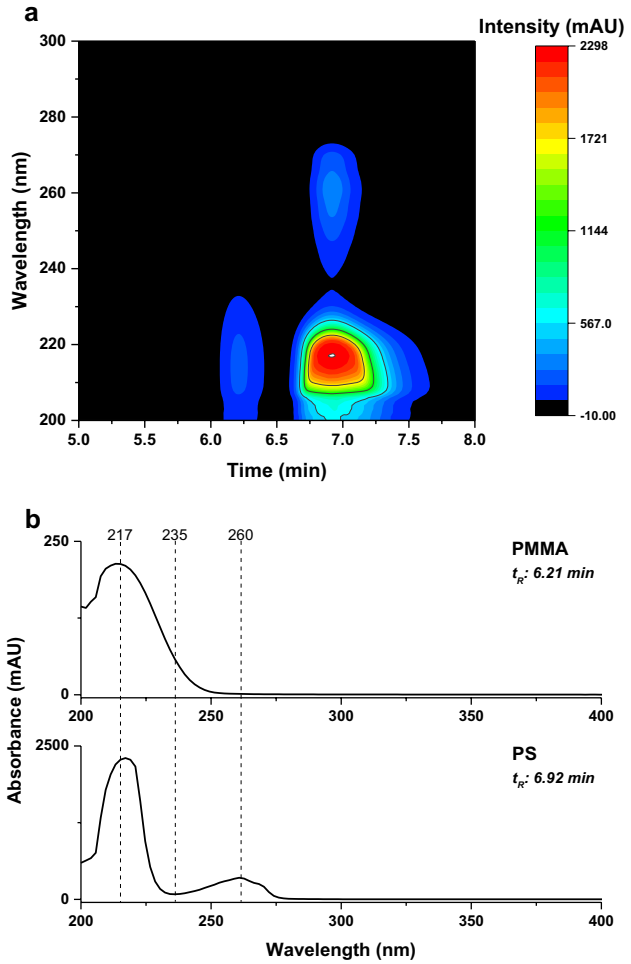

Fig. 1 **a** 3DFIELD chromatogram collected from the SEC/PDA analysis of an ≈1:1 blend of PMMA 838 K and PS 111 K. **b** UV spectra extracted from the 3DFIELD chromatogram for PMMA 838 K at retention time (t_R) of 6.21 min (above) and for PS 111 K at t_R of 6.92 min (below)

Figure 2 shows the SEC/UV chromatograms of the PMMA 838 K + PS 111 K blend taken from the 3DFIELD chromatogram in Fig. 1a, at 235 nm (top) and 260 nm (bottom). At 235 nm, both polymers are observable in the SEC chromatogram, with the larger PMMA eluting earlier than the smaller PS, as expected from a size-exclusion separation. At 260 nm, the only peak present is due to the PS. A comparison of these chromatograms provides an example of *wavelength orthogonality* within a given spectroscopic detection method; in the UV region, PMMA is spectroscopically invisible at 260 nm, but not at 235 nm.

Detection Orthogonality Between Spectroscopic Techniques: SEC/DRI/UV/FL of PS/PMMA and PS/PS-Anth-PS blends

Figure 3 shows the SEC/DRI chromatogram of the PMMA 838 K + PS 111 K blend. Both components of the blend can be observed with the DRI detector, the signal from which, S_{DRI}, is given by [21]:

$$S_{DRI} \propto c \times \left(\frac{\partial n}{\partial c}\right) \quad (1)$$

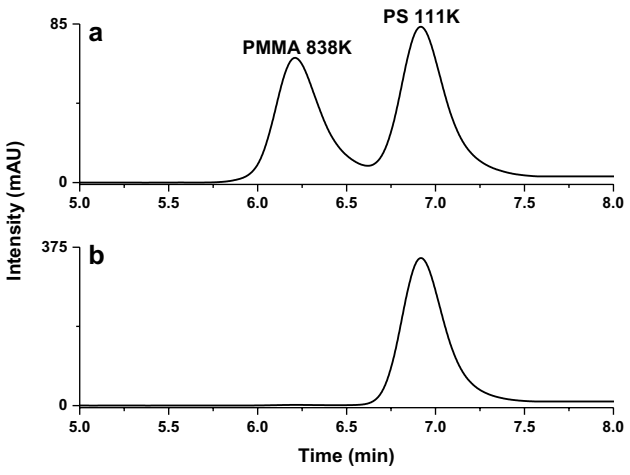

Fig. 2 SEC/PDA chromatograms obtained for an ≈1:1 blend of PMMA 838 K and PS 111 K at **a** 235 nm and **b** 260 nm

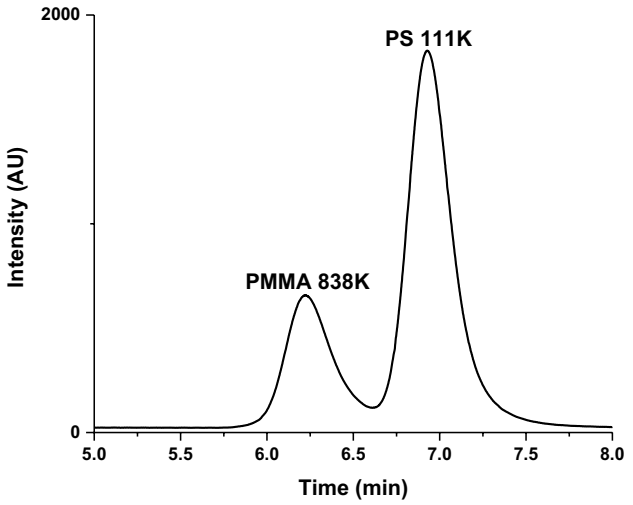

Fig. 3 SEC/DRI chromatogram obtained at a vacuum wavelength of 685 nm for an ≈ 1:1 blend of PMMA 838 K and PS 111 K

where c is the concentration of analyte in solution and $\partial n/\partial c$ is the specific refractive index increments of the solution. The latter term, which can be considered the refractometric analog of the absorptivity in Beer's law, is sample-, solvent-, wavelength-, and temperature-dependent. A recent paper in this journal explores the $\partial n/\partial c$ parameter in detail, with extensive tabulations of experimentally determined values [22]. At experimental conditions identical to those employed here, the $\partial n/\partial c$ of PS was determined to be (0.194 ± 0.004) mL g^{-1} and that of PMMA to be (0.0853 ± 0.0015) mL g^{-1} [22, 23]. It is this difference in $\partial n/\partial c$ between PS and PMMA that is responsible for the larger peak area of PS 111 K as compared to the peak area of PMMA 838 K, given that both components are present at nearly equal concentrations. (It should be noted that the ratio of peak areas is nearly identical to the concentration-normalized ratio of $\partial n/\partial c$ values).

As can be seen when comparing Fig. 3 to Fig. 2b, the two spectroscopic techniques, DRI and UV (260 nm), can be considered orthogonal to each other as regards to PMMA. Indeed, the fact that these are both concentration-sensitive detectors, but the DRI responds to both PS and PMMA while UV (260 nm) responds only to PS, can be used not only for selective detection of components in a blend but also to determine the relative percentages of the two components across the molar mass distribution of a PS-PMMA copolymer [17, 18]. Similar detector sensitivities have also allowed for the recent study of copolymers of styrene and *t*-butyl methacrylate [24].

We next examine a blend of two different polystyrenes, one (PS-Anth-PS 108 K) center-labelled with anthracene, the other unlabeled (PS 3.5 M). Given that anthracene itself naturally fluoresces in the range of 370–460 nm [25], it seems reasonable to assume that solutions of anthracene-labelled PS in THF should also fluoresce. A search of the literature, however, provided no emission or excitation wavelengths or spectra for either "neat" anthracene or PS-labelled anthracene in THF, at any temperature. Therefore, our first task was to determine the optimal excitation and emission wavelengths for the labelled PS, assuming these exist, as described in the Experimental. As shown in Fig. 4, our fluorescence experiments demonstrate that 256 nm and 419 nm correspond, respectively, to the maxima in the excitation and emission spectra of PS-Anth-PS 108 K.

Figure 5a shows the SEC/DRI chromatogram of an ≈ 1:1 blend of PS 3.5 M and PS-Anth-PS 108 K. Both peaks are

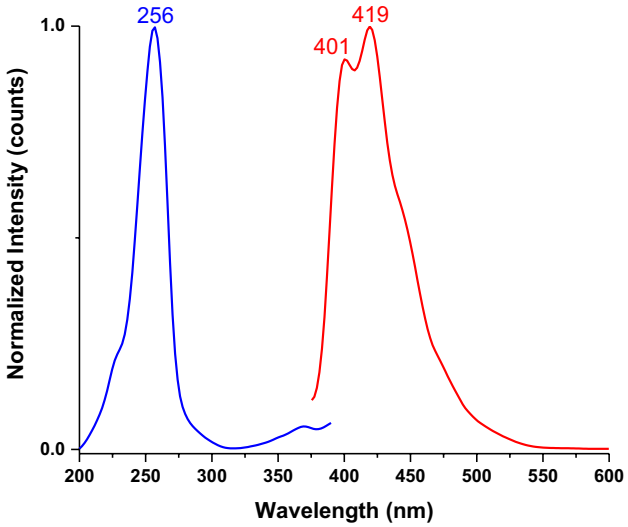

Fig. 4 Fluorescence excitation (blue) and emission (red) spectra collected for PS-Anth-PS 108 K

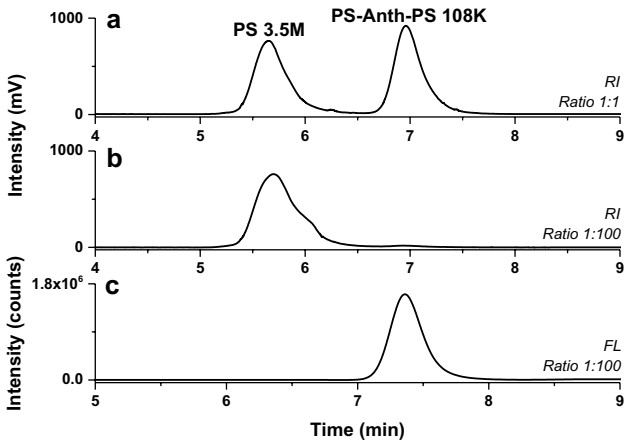

Fig. 5 **a** SEC/DRI (685 nm) chromatogram for an ≈ 1:1 blend of PS-Anth-PS 108 K and PS 3.5 M. **b** SEC/DRI (685 nm) and **c** SEC/FL ($\lambda_{exc} = 256$ nm, $\lambda_{em} = 419$ nm) chromatograms for an ≈ 1:100 blend of PS-Anth-PS 108 K and PS 3.5 M

present in the chromatogram, with the larger PS 3.5 M eluting prior to the smaller, labelled polymer, and with similar peak areas. When the blend contains only ≈ 1% of PS-Anth-PS 108 K, this polymer goes almost unnoticed with the DRI (Fig. 5b). This is because the latter, as given by Eq. (1), is a concentration-sensitive detector which, all other things being equal (which they are), generates a larger response the larger the solution concentration of a sample and, vice versa, the DRI response will be smaller when the solution concentration is smaller (as in the present case).

When the 100:1 blend of unlabeled-to-labeled PS is viewed through the lens of SEC with on-line FL detection, however, a very different picture emerges from that obtained with the DRI. As seen in Fig. 5c, even though PS-Anth-PS 108 K constitutes only ≈ 1% of the blend, its peak is observed by SEC/FL with excellent signal-to-noise ratio (S/N ≈ 1.6×10^6). This sensitivity is made even more remarkable by the fact that the anthracene group is present in only ≈ 1 part-per-thousand of the repeat units of PS-Anth-PS 108 K. Moreover, no FL response is generated by the unlabeled PS 3.5 M, even though it constitutes ≈ 99% of the blend; the PS without an anthracene group is spectroscopically invisible to FL detection. This example showcases the sensitivity of fluorescence detection to even very minor components in a sample, as long as these components possess a fluorescent moiety of high fluorescent efficiency (high fluorescence quantum yield). It also serves to demonstrate the orthogonality of DRI and FL detection with respect to fluorescent/non-fluorescent components of a sample.

Detection Orthogonality of Coeluting Blend Components

Figure 2b shows the SEC/UV chromatogram at 260 nm of an ≈ 1:1 blend of PMMA 838 K and PS 111 K. One way to know that there are two components in the blend is by looking at the SEC/DRI chromatogram in Fig. 3: Both the PS and the PMMA elicit a DRI response and separate from each other because of their different hydrodynamic volumes (different sizes in solution). The question then arises as to how to determine if more than one component is present when the components of a blend each have the same, or very similar, hydrodynamic volumes, meaning that the components would not be separated from each other in an SEC experiment (nor, as mentioned in the Introduction, would they be separated in other size-based separations such as hydrodynamic chromatography of flow field–flow fractionation). This type of coelution has rarely been investigated in size-based separations, though it likely occurs quite often in the analysis of copolymers, complex polymers, and blends, falling more generally within the purview of so-called interaction polymer liquid chromatography techniques (e.g., gradient polymer elution chromatography [26, 27] or temperature gradient interaction chromatography [28]).

To answer the question posed above, in a one-dimensional size-based separation, requires some a priori knowledge of the sample or, at least, of one of its components. Let us take, for example, a blend of PS 111 K and PMMA 107 K, both of which have nearly identical sizes in solution in THF at room temperature. Were we unaware of the spectroscopic invisibility of PMMA in the UV at 260 nm, we would also then be unaware of whether the peak in the SEC/UV chromatogram at this wavelength is due to a single component or to two components coeluting because of their coincidence in size. An SEC/UV chromatogram (260 nm) of PS on its own would not inform our knowledge in this matter.

Let us suppose we have independent knowledge that PS 111 K is one of the components of the blend. From the SEC/DRI trace of this polymer (Fig. 3), we see a peak area of ≈ 519 AU min. In Fig. 6 we see that, for the same solution concentration of PS 111 K and same injection volume as those employed to generate Fig. 3, the SEC/DRI peak now has an area of ≈ 784 AU min. This difference in peak area provides a clear indication that "something" is coeluting with PS 111 K in the SEC experiment. (Concluding that the coeluting species is a PMMA, based solely on the fact that the difference in peak areas corresponds to the peak area of a 1 mg mL^{-1} solution of PMMA, is not wise, as many polymers have similar $\partial n/\partial c$ to PMMA and, consequently, many polymers could account for a similar peak area difference).

Similar reasoning to that employed above when comparing SEC/UV at 260 nm to SEC/DRI for blends of coeluting species where one species in spectroscopically invisible in the UV can be employed when comparing SEC/UV traces at different wavelengths for the same blend. In the latter case,

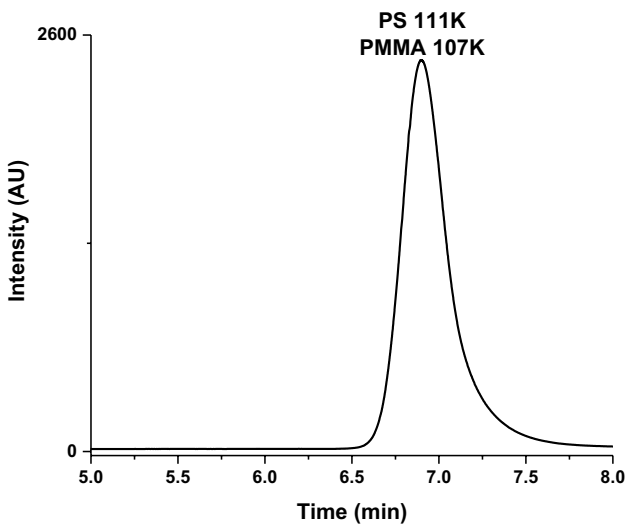

Fig. 6 SEC/DRI chromatogram for an ≈ 1:1 blend of PMMA 107 K and PS 111 K

the wavelength orthogonality described earlier can be capitalized upon to help determine if more than one component is present in a peak, given that a priori knowledge about one of the components in the blend exists.

Conclusions

In an earlier publication [5], it had been shown how a detection method such as viscometry, which relies on hydrodynamic transport properties of polymer solutions, provides information orthogonal to that provided by spectroscopically based detectors such as the refractometer or different types of light scattering photometers. Here, it has been shown how different spectroscopic techniques can provide information orthogonal to one another, namely refractometry with fluorescence and refractometry with UV absorption. Moreover, within a given spectroscopic technique such as UV, wavelength orthogonality can provide a means by which to discriminate amongst sample components, allowing detection of components at one wavelength which are spectroscopically invisible at a different wavelength.

Employing SEC with on-line refractometry, UV absorption, and FL detection (SEC/DRI/UV/FL), blends of PS and PMMA were studied. The lack of absorption by PMMA at 260 nm means that this polymer is spectroscopically invisible in the UV at this wavelength. PMMA can be observed, however, either when employing DRI detection or when monitoring a different UV wavelength (e.g., 217 nm or 235 nm). This same type of spectroscopic invisibility is observed for the non-fluorescent component(s) of a blend when employing on-line FL detection. The sensitivity of the latter method was showcased here by its ability to detect, with a $S/N \approx 1.6 \times 10^6$, the fluorescent component of a blend when this component comprised only ≈ 1 % of the blend (with the fluorescent group comprising only ≈ 1 part-per-thousand of the labeled polymer's repeat units).

In blends with PS of different size than PMMA, DRI detection or UV detection at either 217 nm or 235 nm show two distinct SEC peaks, whereas the SEC/UV trace at 260 nm shows only one peak, that due to PS. In the less-explored case of blends composed of PS and PMMA of the same size which, therefore, coelute in an SEC experiment, only one peak is observed. If, however, a priori knowledge about one of the components of the blend exists, the peak areas obtained by SEC/DRI or SEC/UV at 217 nm or 235 nm can be indicative of sample component coelution.

We hope that the present experiments continue to shed light on the various types of detector orthogonalities that exist among common macromolecular separations detection methods (and niche methods, such as FL), and how these methods can complement one another to provide a clearer, more complete picture of the composition of complex polymers and blends.

Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

Research Involving Human Participants and/or Animals This article does not contain any studies with human participant or animals performed by any of the authors.

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