



# On-line coupling of hollow-fiber flow field-flow fractionation and depolarized multi-angle static light scattering (HF5/D-MALS). Proof of principle

André M. Striegel

Chemical Sciences Division, National Institute of Standards and Technology (NIST), 100 Bureau Drive, MS 8390, Gaithersburg, MD 20899-8390, USA

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## ABSTRACT

Introduced here is the on-line coupling of hollow-fiber flow field-flow fractionation (HF5) to depolarized multi-angle static light scattering (D-MALS). HF5 is a size-based separation alternative to size-exclusion and hydrodynamic chromatography and asymmetric flow field-flow fractionation. HF5 can separate larger sizes than its chromatographic counterparts and provides several advantages over its fractionation counterpart, including reduced sample consumption and greater ease of operation. D-MALS is a variant of MALS in which the depolarized scattering from the analyte solution is measured at a variety of angles simultaneously. Measurements of depolarized scattering have previously been employed in studying the optical properties of solutions or suspensions, to determine the length of rod-like analytes, and to gain increased accuracy in the determination of analyte molar mass. The coupling HF5/D-MALS allows for the depolarization ratio of a solution or suspension to be measured continuously across the fractogram. This is demonstrated here for a Teflon latex the size range of which extends beyond that accessible to commercial size-exclusion columns. The results presented provide the first reported on-line HF5/D-MALS coupling, showing the feasibility of the technique as well as its realized potential for providing continuous depolarization measurements, *inter alia*.

## 1. Introduction

Size-based separations are among the most used methods for macromolecule characterization. Among these techniques, size-exclusion chromatography (SEC) is employed far more than any other [1]. SEC is not without its limitations, however [2], one of these being the analysis of very large and/or very fragile analytes. In this limiting regime, other techniques such as hydrodynamic chromatography (HDC) and flow field-flow fractionation (flow FFF) can often be successfully employed [3-5]. Variants of the latter include the more popular asymmetric flow FFF (AF4) and the generally underutilized hollow-fiber flow FFF (HF5) [6,7].

In HF5, solutions or suspensions flow through a hollow fiber with semi-permeable walls encased within a solid housing. Suction is applied concentrically to the fiber, causing analytes to migrate to and focus on the fiber walls, the latter being the FFF accumulation wall. As in AF4, cross-flow programming can be employed in HF5 to improve resolution and to ensure retention and elution within a given timeframe and within the given volume constraints of the hollow fiber.

Some potential advantages of HF5 vis-à-vis AF4 are the possibility of

achieving higher sensitivity in the former, as a result of lower analyte dilution because of the smaller volume in the hollow fiber versus in an AF4 channel; a lower propensity for overloading and, thus, for analyte aggregation in HF5, given the much lower concentrations employed, about one-third those employed in AF4; and a less complicated experimental set-up, as HF5 cartridges are easily installed and removed as a single unit, as opposed to the assembly and disassembly required for membrane installation and replacement in AF4 channels. One notable drawback of HF5 is its generally lower signal-to-noise ratios ( $S/N$ ) as compared to AF4, due to the lower concentrations employed in HF5 and the lower amount of analyte injected in this technique [8].

Among the most popular on-line detection methods for macromolecular separations is multi-angle static light scattering (MALS). With an accompanying concentration-sensitive detector, MALS is normally employed to determine the molar mass and size of a sample [1,9]. A less popular use for MALS has been to study the depolarization state of dilute polymer solutions, as to do this requires the use of depolarized MALS (D-MALS). This latter detection method offers the possibility of observing the angular dependence of the optical behavior of dilute polymeric or colloidal solutions, of calculating the length of rod-like

E-mail address: [andre.striegel@nist.gov](mailto:andre.striegel@nist.gov).

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objects, and more, including allowing for more accurate calculations of analyte molar mass and of the Rayleigh factor of solvents. D-MALS differs from depolarized dynamic light scattering. The former measures the depolarized time-averaged fluctuations of the light scattered by a dilute analyte solution. The latter measures the depolarized time-dependent fluctuations of this scattered light and is used to disambiguate from each other the translational and rotational diffusion coefficients of analytes and to assist when evaluating the angular dependence of decay rates in dynamic light scattering, among other applications.

On-line D-MALS was introduced as a detection method for SEC over two decades ago and applied to a variety of macromolecular scenarios [10,11]. Its use since appears to have languished, however. With the aim of reintroducing this detection method to a new generation of separation scientists, coupled to a technique which extends into a higher size regime than does SEC, we have attempted the on-line coupling of HF5 to D-MALS. The principles of D-MALS are explained in [12] within the context of on-line detection and, in more fundamental but off-line, batch-mode fashion, also in [13]. As such, only a brief introduction is given here. Our test analyte is a Teflon emulsion with average diameter ranging from 0.1  $\mu\text{m}$  to 0.5  $\mu\text{m}$  [14], i.e., with an upper size limit generally exceeding that of commercially available SEC columns [15]. We hope that this demonstration of the feasibility of performing on-line HF5/D-MALS will encourage others to attempt these types of experiments for their own purposes.

## 2. Experimental

### 2.1. Materials

$\text{Na}_2\text{HPO}_4$  (Sigma-Aldrich, St. Louis, MO),  $\text{NaN}_3$  (J.T. Baker, Phillipsburg, NJ, USA), Brij-35 (Honeywell-Fluka, Morris Plains, NJ), sodium dodecyl sulfate (Polysciences, Warrington, PA, USA). The Teflon latex employed was Algoflon #D60 V (Solvay, Baltimore, MD, USA), kindly provided by Paul Russo (Georgia Institute of Technology) and Rafael Cueto (Louisiana State University).

### 2.2. HF5/D-MALS analysis

The HF5 analyses were carried out using an Agilent 1260 isocratic HPLC pump and autosampler (Agilent Technologies, Santa Clara, CA, USA), an Eclipse DualTec FFF control module (Wyatt Technology Co., Santa Barbara, CA, USA), and an on-line MALS photometer (DAWN HELEOS-II, Wyatt). While an on-line DRI detector (T-rEX, Wyatt) was also part of the system, injected concentrations were too low to yield usable concentration data.

The HF5 cartridge contained a 17-cm-long polyethersulfone fiber of  $\approx 0.8$  mm inner diameter and 1.3 mm outer diameter, with a nominal cut-off of 10 kDa (Wyatt). The HF5 analysis conditions are summarized in Table 1. A linearly decaying cross-flow starting from 0.2  $\text{mL min}^{-1}$  was employed. All the analyses were carried out in triplicate using Milli-Q (Millipore, Billerica, MA, USA)  $\text{H}_2\text{O} + 0.02\%$   $\text{NaN}_3 + 0.002$  mol  $\text{L}^{-1}$   $\text{HNa}_2\text{PO}_3 + 0.2\%$  Brij-35 + 0.05 % sodium dodecyl sulfate, an eluent previously used successfully for the HDC separation of various types of latexes [16,17]. Detectors were temperature-regulated at  $(25.0 \pm 0.1)^\circ\text{C}$ , while the HF5 fiber temperature was ambient. Fiber flow was

**Table 1**  
HF5 analysis conditions

Mode	Duration (min)	Cross-flow start ( $\text{mL min}^{-1}$ )	Cross-flow stop ( $\text{mL min}^{-1}$ )	Flow profile
Elution	2.0	0.2	0.2	Constant
Focus	1.0	0.5	0.5	Constant
Focus + inject	3.0	0.5	0.5	Constant
Elution	25.0	0.2	0.0	Linear

1  $\text{mL min}^{-1}$ ; injection volume was 5  $\mu\text{L}$ .

On-line D-MALS employed the same MALS detector as described above, which generates a vertically polarized laser beam. In this detector, odd-numbered photodiodes are located on one side of the cell and even-numbered ones on the other. For D-MALS, two strips of Polaroid film (Wyatt), one with vertical polarization axis and one with horizontal polarization axis, are placed around the sides of the MALS flow cell and maintained tightly in place by a grooved cell retainer attached to the cell. The strips thus play the role of analyzer in the optical train of the system. Two sets of experiments are performed, first with the vertically polarized Polaroid strip facing the odd-numbered photodiodes and the horizontally polarized strip facing the even-numbered ones. The position of the strips is then reversed, and the experiment is repeated. Great care must be taken during installation and removal of the strips, to ensure their snugness around the cell, to avoid or at least minimize “leaking” of light around the strips as well as kinking of the strips, and to avoid touching them or the MALS flow cell with one’s fingers (the use of soft-tipped tweezers is recommended, as is an anti-static strap). It should be noted that the same dissolutions of sample were analyzed in both modes, i.e., with both Polaroid strip positions, as well as without the strips in place.

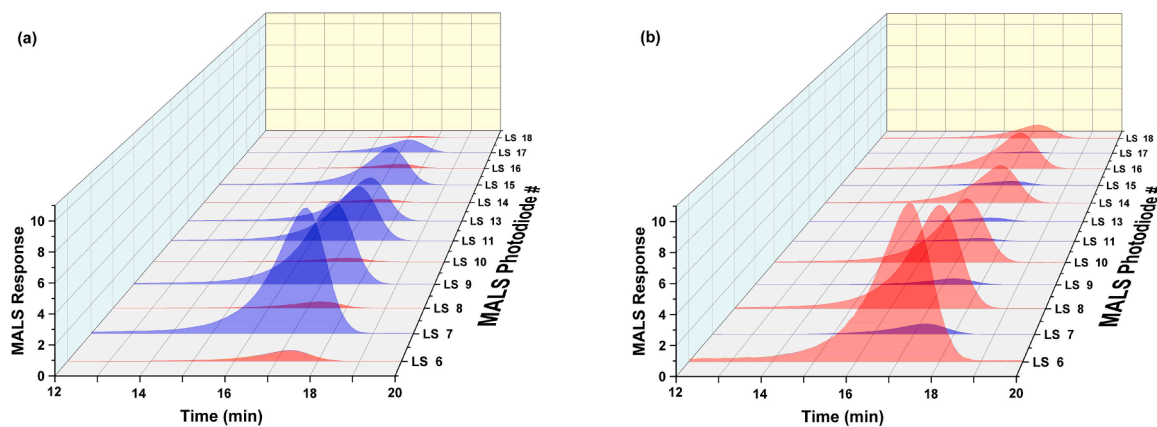
## 3. Results and discussion

For a completely isotropic analyte, the dipole induced by interaction with incident radiation will be parallel to the electric vector  $E_0$  of said radiation. If the analyte is non-absorbing, then only the component of the scattered light which is parallel to the transmission axis of the analyzer (in the present case, the Polaroid strips),  $E_0 \cos \phi$ , will pass through the strips onto the MALS photodiodes. (The angle  $\phi$  is that between the polarization and transmission axes). The intensity  $I(\phi)$  of the scattered light reaching the photodiodes is given by [18]:

$$I(\phi) = \frac{c\epsilon_0}{2} E_0^2 \cos^2 \phi \quad (1)$$

where  $c$  is the speed of light in vacuum and  $\epsilon_0$  is the permittivity of free space. The intensity of the scattered light which is transmitted by the analyzer reaches a maximum,  $I_{\max} = (c\epsilon_0 E_0^2)/2$ , when  $\phi = 0^\circ$ ; conversely,  $I = 0$  when  $\phi = 90^\circ$ . When an isotropic analyte in the MALS flow cell scatters the vertically polarized incident laser light, all of the scattered light should then reach the photodiodes facing the Polaroid strip with vertical polarization axis, and none of the scattered light should reach the photodiodes facing the strip with horizontal polarization axis. Depolarizing analytes will alter these relations, displaying anywhere from mild to strong (depending on the system) scattering in so-called “cross-polarization” mode, i.e., the intensity of scattered light reaching the photodiodes facing the Polaroid strip with horizontal polarization axis will be greater than zero (when vertically polarized incident radiation is employed, as is the case here). It should be noted that, in reality, not all the scattered light will reach the photodiodes in “aligned-polarization” mode (i.e., when the transmission axis of the analyzer is aligned with the polarization axis of the incident radiation), due to an approximately 10 % to 20 % absorption of light by the Polaroid filters. An *a posteriori* route for correcting for this absorption during data treatment, derived for SEC/D-MALS but also potentially applicable to other types of on-line D-MALS experiments, was presented in [11].

Results from our HF5/D-MALS experiments are shown in Fig. 1. Panel (a) shows the results when the experiments were performed in aligned-polarization mode for the odd-numbered photodiodes, cross-polarization mode for the even-numbered ones. (Note that data for photodiode 12 are missing, as this photodiode was removed from the instrument to make room at this location for a different, unrelated type of experiment. Results for photodiodes 2 through 5 are absent either because of “dead” photodiodes that needed replacement or because of excessive noise in the data, rendering the latter unusable. As seen,



**Fig. 1.** Results from on-line HF5/D-MALS analysis of dilute Teflon emulsion. (a) With odd-numbered MALS photodiodes in aligned-polarization mode, even-numbered photodiodes in cross-polarization mode. (b) Reverse situation to that in panel (a). Nominal angular range of measurement spans from  $50.0^\circ$  (photodiode #6) to  $147.0^\circ$  (photodiode #18). Photodiode #11 is located at  $90.0^\circ$ . All angles are relative to the direction of the incoming laser beam in the MALS unit.

however, usable data from twelve different photodiodes were obtained). The HF5/D-MALS fractograms as measured by the odd-numbered photodiodes, in blue, are substantially larger (greater intensity or, more accurately, greater  $S/N$ ) than are the fractograms measured by the even-numbered photodiodes, in red. The opposite relations are observed in Fig. 1b, where the odd-numbered photodiodes now measure the scattered light intensity in cross-polarization mode and the even-numbered photodiodes the intensity in aligned mode.

In both panels (a) and (b) of Fig. 1, the fractograms obtained at various angles in cross-polarization mode, while of lower  $S/N$  than their aligned-mode counterparts, are still evident, with the measured scattering most definitely being non-zero. This provides evidence that, to some degree, the Teflon particles have depolarized the scattered light at these angles. This result is given more quantitative meaning next.

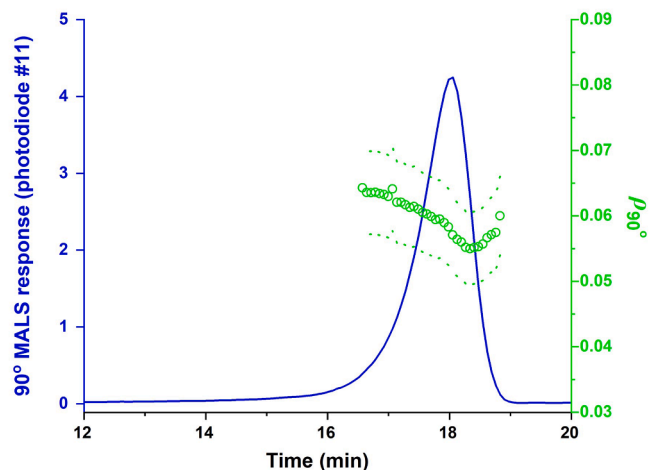
Most macromolecules and particles are weak depolarizers. Teflon particles, on the other hand, can be considered strong depolarizers, though only relative to those former cases. A metric by which to quantify this is the depolarization ratio  $\rho_\theta$ , defined as the ratio of the scattering intensity in cross- versus aligned-polarization mode at a given scattering angle  $\theta$  [10-12,19]:

$$\rho_\theta \equiv \left( \frac{I_{Hv}}{I_{Vv}} \right)_\theta \quad (2)$$

where  $I$  denotes the scattered light intensity, “H” and “V” the direction (horizontal or vertical, respectively) of the transmission axes of the Polaroid filters (analyzers), and “v” the polarization state (vertical) of the incident radiation. Fig. 2 shows an overlay of  $\rho_{90^\circ}$ , obtained by ratioing the intensity of light measured by the  $90^\circ$  MALS photodiode (photodiode #11) in  $Hv$  and  $Vv$  modes, across the HF5 peak of the Teflon particles as determined by  $90^\circ$  D-MALS ( $Vv$  mode). While a decrease in the depolarization ratio across the peak is observed (remembering that, in HF5, smaller analytes elute ahead of larger ones), this change is quite modest, from 0.064 to 0.055. These values are in good agreement with those obtained by Collins et al., in a somewhat different aqueous carrier solution than that employed here [14]. Those authors measured a depolarization ratio of  $0.052 \pm 0.003$  using off-line, batch-mode light scattering, with the ratio being essentially invariant to analyte size for six different fractions. As can be seen in Fig. 2, one advantage of on-line D-MALS is the ability to measure the depolarization ratio continuously across an analyte’s fractogram peak.

#### 4. Conclusions

Demonstrated here is the feasibility of the on-line coupling of hollow-fiber flow field-flow fractionation (HF5) to depolarized multi-



**Fig. 2.** Depolarization ratio  $\rho_{90^\circ}$  (open green circles) as a function of HF5 elution time for Teflon particles analyzed. Fractogram (solid blue line) as obtained by  $90^\circ$  D-MALS in aligned-polarization ( $Vv$ ) mode. Dashed green lines represent 1 standard deviation from the mean, based on triplicate measurements.

angle static light scattering (D-MALS). The HF5/D-MALS set-up was employed to analyze dilute emulsions of Teflon of a size far too large for analysis by SEC employing commercially available columns. Results from our analysis show that the Teflon particles depolarize scattered light non-negligibly at all angles of observation. The measured  $90^\circ$  depolarization ratio is in good agreement with discrete values previously reported in the literature, while the coupling of D-MALS to HF5 allowed for this ratio to be measured continuously across the analyte’s fractogram peak.

HF5 has already proven itself a good alternative to other size-based separation methods, reaching a higher size regime than, e.g., SEC or HDC, and being, *inter alia*, operationally simpler and less sample-demanding than its flow FFF counterpart AF4. On-line D-MALS, on the other hand, requires more user involvement than does “simple” MALS, in the careful installing and removing of Polaroid strips from around the light scattering detector cell and in the careful handling of this cell. It is hoped that these difficulties will be offset by the great potential of HF5/D-MALS for studying the depolarization of dilute polymer and particle solutions, continuously as a function of size and discretely as a function of scattering angle. These measurements can serve to better inform our knowledge of solution properties, of analyte size, and to provide for increased accuracy in molar mass and Rayleigh factor determinations

when employing on-line static light scattering detection [20,21].

### CRediT authorship contribution statement

**André M. Striegel:** Conceptualization, Investigation, Formal analysis, Writing – original draft, Writing – review & editing.

### Declaration of competing interest

The author declares no financial interests or personal relationships that would influence the work reported in this paper.

### Data availability

All relevant data are included.

### Disclaimer

Commercial products are identified to specify the experimental procedure adequately. 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. The views expressed in this article are those of the author and do not necessarily reflect the official policy or position of the National Institute of Standards and Technology.

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