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Article

Method for Improved Fluorescence Corrections for Molar Mass Characterization by Multiangle Light Scattering

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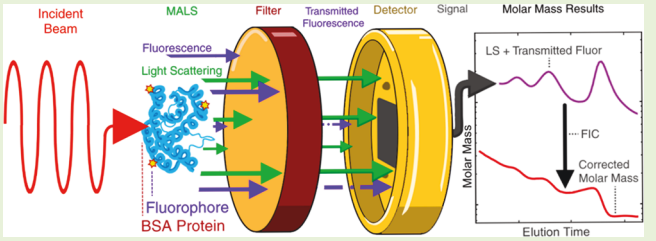
ABSTRACT: Multiangle light scattering (MALS) was used to determine the absolute molar mass of fluorescent macromolecules. It is standard protocol to install bandwidth filters before MALS detectors to suppress detection of fluorescent emissions. Fluorescence can introduce tremendous error in light scattering measurements and is a formidable challenge in accurately characterizing fluorescent macromolecules and particles. However, we show that for some systems, bandwidth filters alone are insufficient for blocking fluorescence in molar mass determinations. For these systems, we have devised a correction procedure to calculate the amount of fluorescence interference in the filtered signal. By determining the intensity of fluorescent emission not blocked by the bandwidth filters, we can correct the filtered signal accordingly and accurately determine the true molar mass. The transmission rates are calculated before MALS experimentation using emission data from standard fluorimetry techniques, allowing for the characterization of unknown samples. To validate the correction procedure, we synthesized fluorescent dye-conjugated proteins using an IR800CW (LI-COR) fluorophore and Bovine Serum Albumin protein. We successfully eliminated fluorescence interference in MALS measurements using this approach. This correction procedure has potential application toward more accurate molar mass characterizations of macromolecules with intrinsic fluorescence, such as lignins, fluorescent proteins, fluorescence-tagged proteins, and optically active nanoparticles.



INTRODUCTION

When light propagates through a medium, it creates an oscillating electric field that induces oscillating dipoles in molecules that it passes through. The oscillating dipoles within molecules create electromagnetic fields—thus emitting light, a phenomenon known as light scattering (LS). Our focus lies with elastic light scattering (Raleigh scattering), where there is no change in the wavelength of the emitted light from the incident light. Most importantly, Raleigh scattering can be used to determine the molar mass of a molecule. The relationship between molar mass and scattered light of molecules in a dilute solution when using the Raleigh-Gan-Debye approximation[1] (RGD) is given.

$$R_\theta = K^* McP(\theta)[1 - 2A_2McP(\theta)] \quad (1a)$$

$$K^* = 4\pi^2 n_0^2 \frac{(dn/dc)^2}{\lambda^4 N_A} \quad (1b)$$

where R_θ (cm^{-1}) is the excess Raleigh ratio as measured by a detector at angle θ, M is the weight-average molar mass (g/mol), $P(\theta)$ is the form factor, K^* is the optical constant, and c is the concentration. Elaborations on RGD can be found elsewhere in the literature.[1,2] For large molecules ($R_g \geq$ about 10 nm for a 658 nm laser),[1] the scattered light intensity will have an angular dependence, represented in $P(\theta)$. Theoretically, all size and shape effects vanish ($P(\theta) \rightarrow 1$) when intensities are measured at the same angle as the incident beam ($\theta = 0°$), however, measuring scattering at $\theta = 0°$ cannot be done in practice without also measuring the intensity of the incident light.[1] Instead, multiple angles about the illuminated sample can be measured and then extrapolated to 0° to arrive at a size and shape independent of molar mass: this is called multiangle light scattering (MALS). By examining the angular dependence and angular variation of scattered light, we can also determine the radius of gyration and shape, respectively. However, shape information is limited to very large macromolecules. A combination of MALS with an online fractionation technique such as size-exclusion chromatography (SEC-MALS), is often used to characterize disperse solutions and is employed in this study. This technique allows for the additional determination of the number-average molar mass (M_n) and dispersity ($Đ$, M_w/M_n).

Fluorescent macromolecules and nanoparticles are prevalent in biological, chemical, and medical fields, where fluorescent light is emitted and can significantly obscure scattered light

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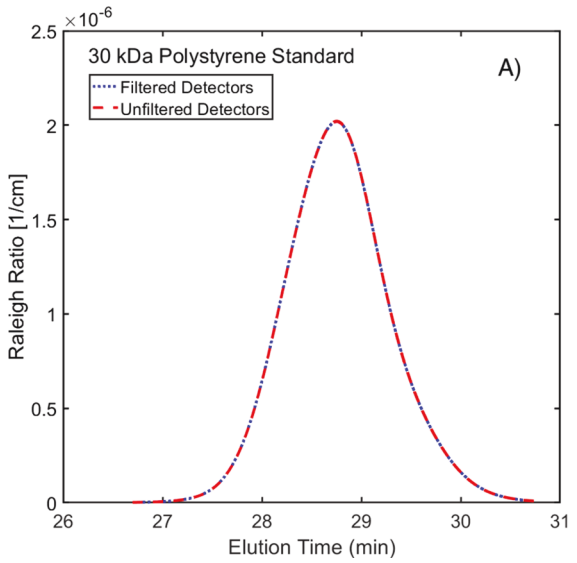
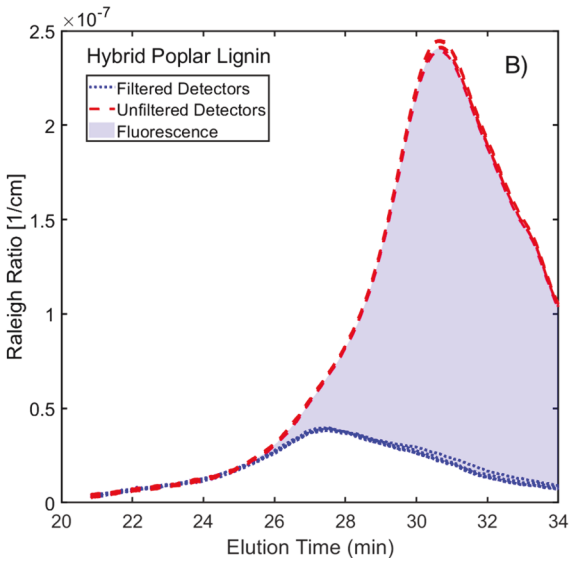
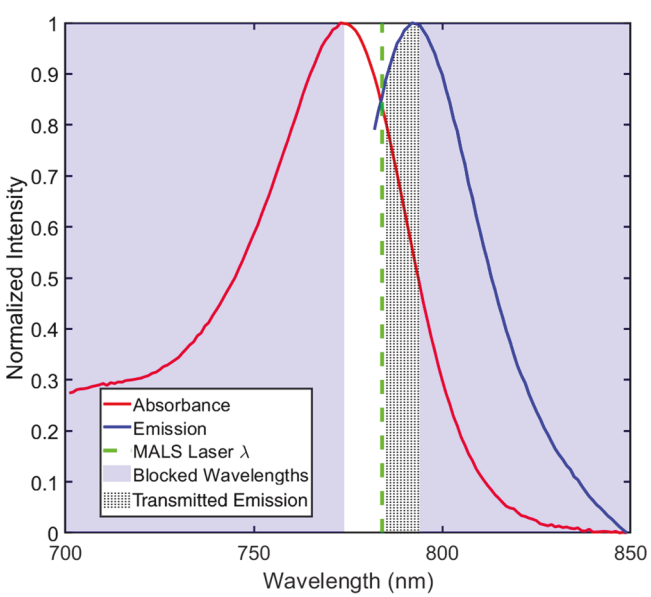

30 kDa Polystyrene Standard
A)
Filtered Detectors
Unfiltered Detectors
Raleigh Ratio [1/cm]
Elution Time (min)


Hybrid Poplar Lignin
B)
Filtered Detectors
Unfiltered Detectors
Fluorescence
Raleigh Ratio [1/cm]
Elution Time (min)

[illegible]

[illegible]


Absorbance
Emission
MALS Laser λ
Blocked Wavelengths
Transmitted Emission
Normalized Intensity
Wavelength (nm)

[illegible]

[illegible]

[illegible]	[illegible]			[illegible]		
[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]

[illegible]

$$R_{\theta} = R_{\theta S} + R_{\theta F}$$

$$R_{\theta F} = N_{\theta} A_{\mathrm{CSCC}} \left[\frac{(V_{\theta F} - V_{\theta,\mathrm{baseline}})}{V_{\mathrm{LM}}} \right]$$

$$R_{\theta S} = N_{\theta} A_{\mathrm{CSCC}} \left[\frac{(V_{\theta S} - V_{\theta,\mathrm{baseline}})}{V_{\mathrm{LM}}} \right]$$

[illegible]

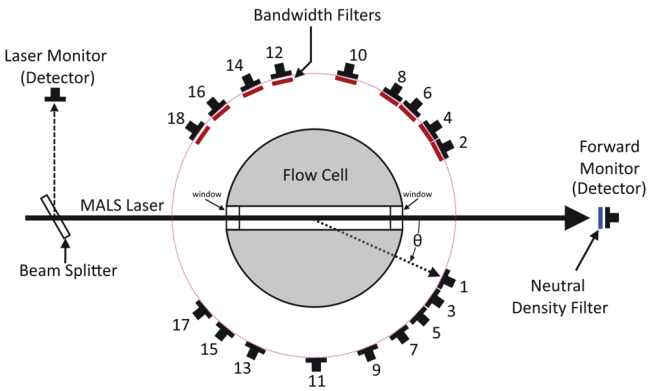
$$R_{\theta,\mathrm{unfiltered}} = R_{\theta S} + R_{\theta F}$$

$$R_{\theta,\mathrm{filtered}} = R_{\theta S} + T_{\mathrm{f}} R_{\theta F}$$

[illegible]

$$R_{\theta S} = \left(\frac{R_{\theta,\mathrm{filtered}}}{1 - T_{\mathrm{f}}} \right) - \left(\frac{T_{\mathrm{f}} R_{\theta,\mathrm{unfiltered}}}{1 - T_{\mathrm{f}}} \right)$$

[illegible]



[illegible]

[illegible]

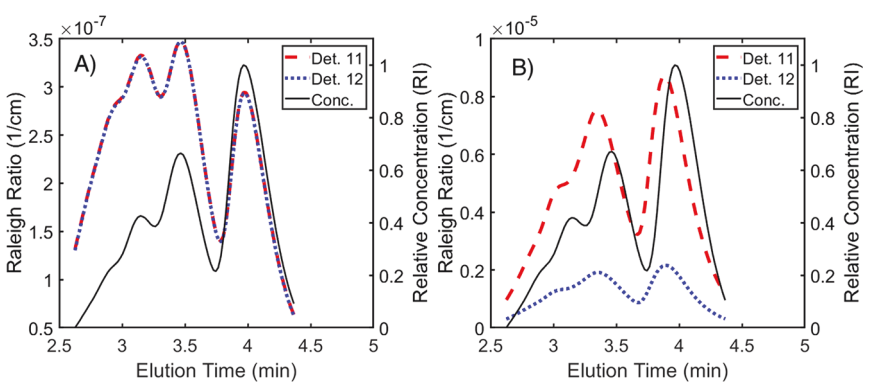
$$R_\theta = N_\theta A_{CSCC}\left[\frac{V_\theta - V_{\theta,\text{baseline}}}{V_{\text{laser}}}\right] \quad (9)$$

[illegible]

5. [illegible]

8. [illegible]

[illegible]

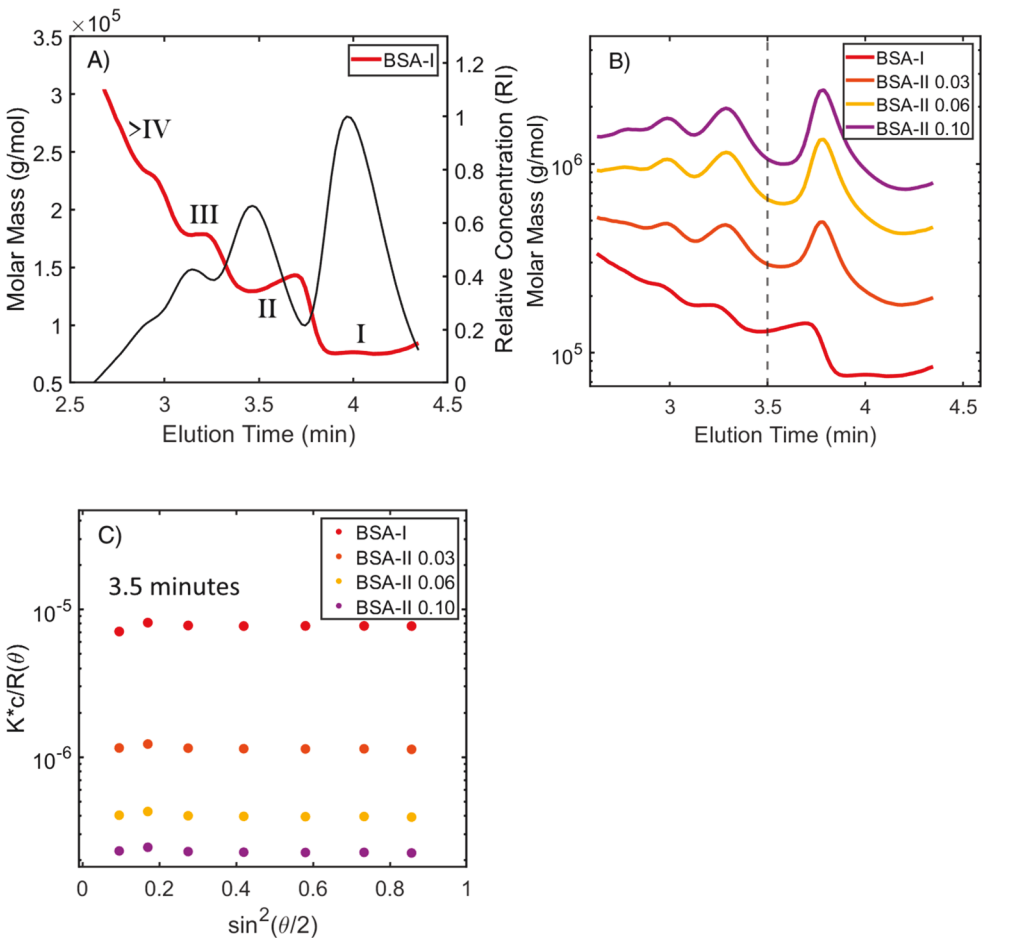


[illegible]

[illegible]

[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
[illegible]	5.B8	58B8B	5%$B	5.B8	58B8B	5%$B			
[illegible].@	5.B8	58B8B	5%$B	8-?B	@85%@	5%$@	5.B8	5@%	5%	8@DD
[illegible].9	5.B%@	5@%	5%$B	9D.%	D.8%$	5%$5	5.B%@	58B$	5%$B	8@%9
[illegible].%.	5.B%@	5@%$	5%$B	55?B	58D9	5%$5	5.B%@	58D%	5%$D	8@9:

[illegible]



[illegible]

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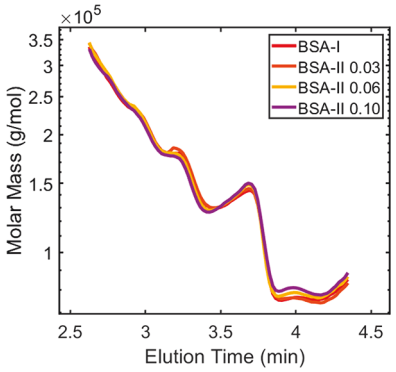
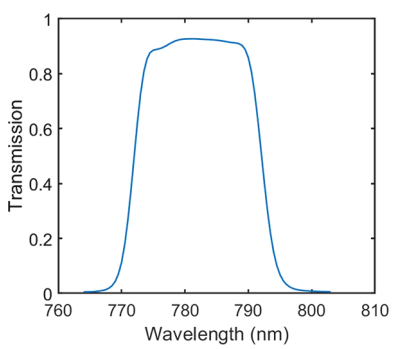
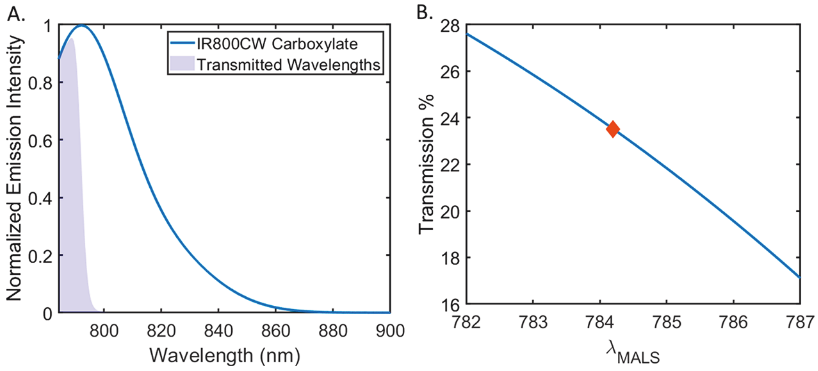
2 [illegible]

[illegible]


×10^5
3.5
3
2.5
2
1.5
1
Molar Mass (g/mol)
2.5
3
3.5
4
4.5
Elution Time (min)
BSA-I
BSA-II 0.03
BSA-II 0.06
BSA-II 0.10


1
0.8
0.6
0.4
0.2
0
Transmission
760
770
780
790
800
810
Wavelength (nm)



A.
Normalized Emission Intensity
IR800CW Carboxylate
Transmitted Wavelengths
Wavelength (nm)
800
820
840
860
880
900
0
0.2
0.4
0.6
0.8
1
B.
Transmission %
λ_{MALS}
782
783
784
785
786
787
16
18
20
22
24
26
28

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