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Are fluorescence-detected sedimentation velocity data reliable?

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ABSTRACT

Sedimentation velocity analytical ultracentrifugation is a classical biophysical technique that is commonly used to analyze the size, shape, and interactions of biological macromolecules in solution. Fluorescence detection provides enhanced sensitivity and selectivity relative to the standard absorption and refractometric detectors, but data acquisition is more complex and can be subject to interference from several photophysical effects. Here, we describe methods to configure sedimentation velocity measurements using fluorescence detection and evaluate the performance of the fluorescence optical system. The fluorescence detector output is linear over a concentration range of at least 1 to 500 nM fluorescein and Alexa Fluor 488. At high concentrations, deviations from linearity can be attributed to the inner filter effect. A duplex DNA labeled with Alexa Fluor 488 was used as a standard to compare sedimentation coefficients obtained using fluorescence and absorbance detectors. Within error, the sedimentation coefficients agree. Thus, the fluorescence detector is capable of providing precise and accurate sedimentation velocity results that are consistent with measurements performed using conventional absorption optics, provided the data are collected at appropriate sample concentrations and the optics are configured correctly.

Sedimentation velocity (SV)[1] analytical ultracentrifugation (AUC) is a widely used and powerful method to characterize the physical properties of macromolecules and macromolecular complexes in free solution [1–5]. In SV experiments, the radial concentration gradients produced in the presence of a centrifugal field are measured in real time using an optical detection system. The most commonly used detectors, currently available on the Beckman Coulter AUC instruments, monitor sample absorbance or refractive index. The noise characteristics and potential sources of systematic errors for these systems have been described [6]. Fluorescence detectors for the AUC have also been developed [7–9] and are now commercially available (AU-FDS [analytical ultracentrifuge fluorescence detection system], AVIV Biomedical, Lakewood, NJ, USA). Fluorescence detection greatly enhances AUC sensitivity and selectivity, and it allows analysis of high-affinity interactions as well as labeled molecules present in complex media such as serum or in the presence of high concentrations of crowding agents [10,11]. However, fluorescence detection introduces several complications into SV measurements. Issues associated with sample labeling [10] and adsorption of proteins at low concentrations have been described [11]. Fluorescence signal intensity can be affected by several photophysical effects [12]. Solvent properties, the local fluorophore environment, and static and dynamic quenching processes all affect the fluorescence emission and, consequently, influence the sensitivity of an AUC measurement. However, these effects remain constant during an experiment and, thus, will not affect the linearity of signal intensity as a function of fluorophore concentration. The emission may also be affected by self- or hetero-association of a labeled macromolecule, potentially resulting in changes in the fluorophore environment or, in the case of self-association of macromolecules labeled with a fluorophore with a small Stokes shift, self energy transfer. Fortunately, simple control experiments can be performed in a fluorimeter to assess potential effects of association state on fluorescence intensity, and this information can be incorporated into fitting models using programs such as SEDANAL [13].

At elevated concentrations, the fluorophore can absorb a significant fraction of the excitation or emission, thereby reducing the fluorescence intensity at the detector. This phenomenon, known as the inner filter effect, leads to undesirable nonlinear responses at higher concentrations. Although corrections can readily be applied for experiments performed in a fluorimeter with right angle detection [12], the situation is more complex in the confocal geometry [14] that is used in the AU-FDS detector, and corrections are

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[1] *Abbreviations used:* SV, sedimentation velocity; AUC, analytical ultracentrifugation; AU-FDS, analytical ultracentrifuge fluorescence detection system; UV, ultraviolet; dsDNA, double-stranded DNA.

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not easily applied. Measurements using a prototype fluorescence detector for the XL-I analytical ultracentrifuge demonstrated linear responses over a decade concentration range of fluorescein, with nonlinear responses at concentrations above 1 μM attributed to the inner filter effect [8]. Nonlinearity at very low (nM) concentrations was also observed and attributed to adsorption of the analyte onto cell components. In a recent study, nonlinear responses were observed in the analysis of a fluorescein-labeled protein in the mid-nanomolar concentration range [15]. However, only two concentrations of a labeled protein were examined.

Instrument-associated systematic errors may also affect AUC data obtained using fluorescence detection [11]. Quantitative analysis of sedimentation velocity measurements is critically dependent on the absence of systematic errors in the data. For example, nonlinearity can distort the boundary shape in SV experiments and lead to underestimates of sedimentation coefficients. Schuck and coworkers recently reported that sedimentation coefficients derived from fluorescence-detected SV experiments are approximately 10% lower than those obtained using conventional absorbance detection [15].

Here, we describe methods to configure the detector for optimal performance and examine whether systematic errors introduced by use of the AU-FDS fluorescence detector influences SV measurements.

Materials and methods

6-Carboxy fluorescein and Alexa Fluor 488 carboxylic acid, succinimidyl ester, were obtained from Life Technology and dissolved in 50 mM Tris (pH 8.0). DNAs were obtained from IDT and dissolved in TE buffer (10 mM Tris and 1 mM EDTA [ethylenediaminetetraacetic acid], pH 8.0). The sequence of the labeled (top) strand is 5′-Alexa Fluor 488-GGAGAACTTCATGCCCTTCGGAT AAGGACTCGTATGTACC-3′, and the sequence of the unlabeled (bottom) strand is 5′-GGTACATACGAGTCCTTATCCGAAGGGCAT-GAAGTTCTCC-3′. The top and bottom strands were annealed at a concentration of 20 μM in analysis buffer (50 mM KPi and 50 mM KCl, pH 6.0) by heating to 90 °C for 1 min and slowly cooling to room temperature. Sample concentrations were measured by absorption spectroscopy using the following extinction coefficients: 6-carboxy fluorescein, ε_{492} = 81,000 M^{-1} cm^{-1}; Alexa Fluor 488, ε_{495} = 71,000 M^{-1} cm^{-1}; top strand, $\varepsilon_{260} = 4.27 \times 10^5$ M^{-1} cm^{-1}; bottom strand, $\varepsilon_{260} = 3.88 \times 10^5$ M^{-1} cm^{-1}.

Analytical ultracentrifugation experiments were performed using Beckman-Coulter XL-I and XL-A analytical ultracentrifuges equipped with AU-FDS fluorescence detectors. Samples were loaded into either SedVel60 two-sector cells (Spin Analytical) with quartz windows, or SedVel50 two-sector cells (Spin Analytical) with sapphire windows. For the fluorescence measurements, the gain and digital multipliers were adjusted to give approximately 3500 counts at the highest concentration. The focus depth was adjusted to the center of the plateau region of the sample. Other fluorescence data collection parameters were maintained at their default values [16]. For the intensity studies, a low rotor speed of 5000 rpm was used to prevent sedimentation. Buffer densities and viscosities were calculated using SEDNTERP [17]. Continuous sedimentation coefficient distributions were generated using SEDFIT [18]. Global analysis of sedimentation velocity data was performed using SEDANAL [13].

Results and discussion

The height of the focus of the AU-FDS detector should be optimized prior to data collection. The user manual suggests that that this procedure be performed on the calibration cell used by the AU-FDS to calibrate radial distance and the angle of the calibration cell relative to the magnet located on the bottom of the rotor [16]. However, we have found it useful to focus on the sample itself. Fig. 1 shows scans of the normalized signal intensity versus focus height for samples of 6-carboxy fluorescein prepared at several concentrations. The signal intensity increases with distance, reaches a plateau, and then decreases slightly. The initial increase is due to the focus moving from within the top window of the cell into the sample. The signal increase is quite broad because of the limited radial resolution of the AU-FDS. The origin of the signal decrease as the focus is moved deeper within the cell is not known but may be due to cutoff of the cone-shaped excitation beam by the cell walls. Interestingly, the shape of the focus scan is dependent on sample concentration. At the highest concentration (2 μM), there is only a narrow range of distance where the intensity is maximal. The width of this maximum increases with decreasing fluorescein concentration, and the scans become superimposable at 200 nM and below. This behavior is consistent with an inner filter effect where the signal becomes increasingly attenuated by absorption of the excitation and emission as the effective pathlength increases with greater focus depth. In fact, the absence of an extended flat maximum in the focus scan is a qualitative indication that the sample concentration is too high and nonlinearity may be present. We suggest that the focus be set to the middle of the maximum in the sample scan. Although contribution of the inner filter effect could be further reduced by moving the focus to shorter distances, placing it at the maximum is preferable because the signal is maximized and, more important, the intensity is insensitive to slight errors in tracking as the sample is scanned radially. Although sloping plateau intensities have been reported using fluorescence detection [11], we have found that the scans are flat when the focus is placed at the sample maximum.

Because different types of AUC cells have different geometries, the sample focus maximum will likely differ from the maximum for the calibration cell. If the two maxima are significantly offset, setting the focus at the sample maximum could result in a low signal for the calibration cell and cause problems in the angular calibration (magnet lock). The calibration cell has a maximum at approximately 1000 μm, well away from the sample maximum of approximately 4500 μm for the SedVel 60K centerpieces. Thus, we have removed approximately 2 mm from the bottom of the calibration centerpiece to move the calibration maximum to

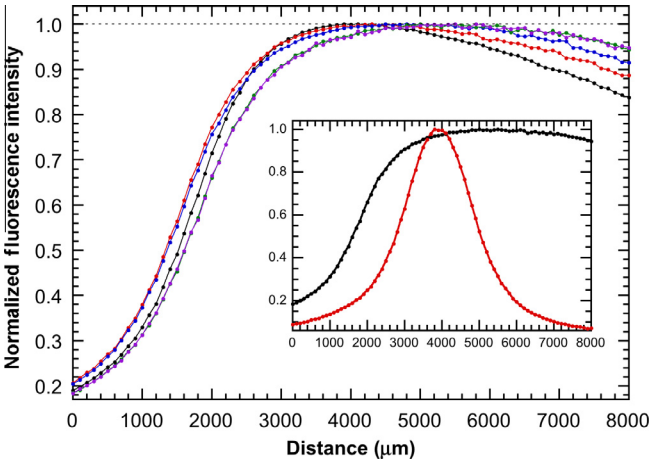

Fig.1. Focus scans. Focus scans of 6-carboxy fluorescein at concentrations of 2 μM (black), 1 μM (red), 500 nM (blue), 200 nM (green), and 100 nM (purple). Inset: Normalized focus scan of the calibration strip (red) and the 200-nM sample (black). Data were collected at 5000 rpm and 20 °C. Scans were normalized to a maximum amplitude of 1. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)