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Demystifying DGE-AUC Part 3: Sample Preparation

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Abstract

This application note is the third in a technical series which will cover the basics of density gradient equilibrium analytical ultracentrifugation or DGE-AUC. In this installment, we discuss the steps involved in preparing samples and setting up a DGE-AUC experiment.

Introduction

Background

In the first installment of this series, we introduced the principles of Analytical Ultracentrifugation (AUC) and discussed its seminal importance in biophysics[1,2]. We then compared several AUC experimental techniques, along with their applications, before introducing a new and orthogonal technique called density gradient equilibrium analytical ultracentrifugation or DGE-AUC. In the second installment of this series, we explored DGE-AUC in more detail, covering data acquisition, visualization and optimization. We have discussed the workflow behind DGE-AUC, in particular, the steps involved in selecting an appropriate gradient forming material (GFM).

In this third installment, we will start by providing a sample protocol to set up a DGE-AUC experiment.

DGE-AUC workflow

In Part II of this technical series, we discussed the workflow to carry out a DGE-AUC experiment, a schematic of which is provided in Figure 1. We will now delve into the details of this workflow in the following pages. Steps involved in sample preparation are enumerated in the following section.

Sample preparation steps for DGE-AUC

Broadly speaking, preparation of a sample for DGE-AUC experiments requires these steps:

1. **Buffer Selection:** Pick a buffer that is chemically compatible with both the target analyte as well as the GFM. This is covered in the preceding installment of this technical series under the heading **Selection of Gradient Forming Material (GFM).**
2. **Prepare stocks of GFM in the buffer.** (See the next section.)
3. **Record UV-Vis absorption of GFM:** (See the next section.)
4. **Record refractive index (RI) of GFM:** (See the next section.)
5. **Prepare stocks of target analyte in the buffer.**
6. **Choose starting density of GFM for experiment.** For instance, this could be: 1.5 g/mL of CsCl.

7. **Choose volume of sample which will be prepared:** A single DGE-AUC in a 2-sector AUC cell requires about ~ 450 µL of sample. Scale up according to number of replicates desired.

8. **Sample preparation:** Preparation of samples for DGE-AUC experiments requires the use of mass and density calculations and *should not be done* by using the standard dilution equation $C_{initial}*V_{initial} = C_{final}*V_{final}$, where C refers to concentration and V refers to volume. Use dilution equations to calculate volumes of stock GFM, stock analyte and buffer needed and prepare samples accordingly. This is discussed in subsequent sections and in Tables 1 through 3.

9. **Record UV-Vis spectrum of samples.** If full spectrum (usually 200 to 750 nm) cannot be recorded, then record the relevant wavelengths (230, 260, 280, 490 nm, etc.).

10. **Record refractive index (RI) of samples.**

11. **Load samples into AUC cells.** (See the next section.)

12. **Set up experiment from remote laptop**/PC from the Optima AUC web interface.

13. **Start experiment. View live data from remote laptop.**

14. **Transfer data from Optima AUC instrument to laptop upon completion of experiment.** Download data as .tar.gz file. Unpack using WinZip or similar software.

15. **Analyze data and generate report.**

The first thing to note from the overall process shown in Figure 1 is that this workflow assumes that the experimentalist already has samples containing premixed target analyte and gradient forming material (GFM). Preparation of these samples requires calculation of suitable stock volumes and dilutions. This is illustrated in the following sections.

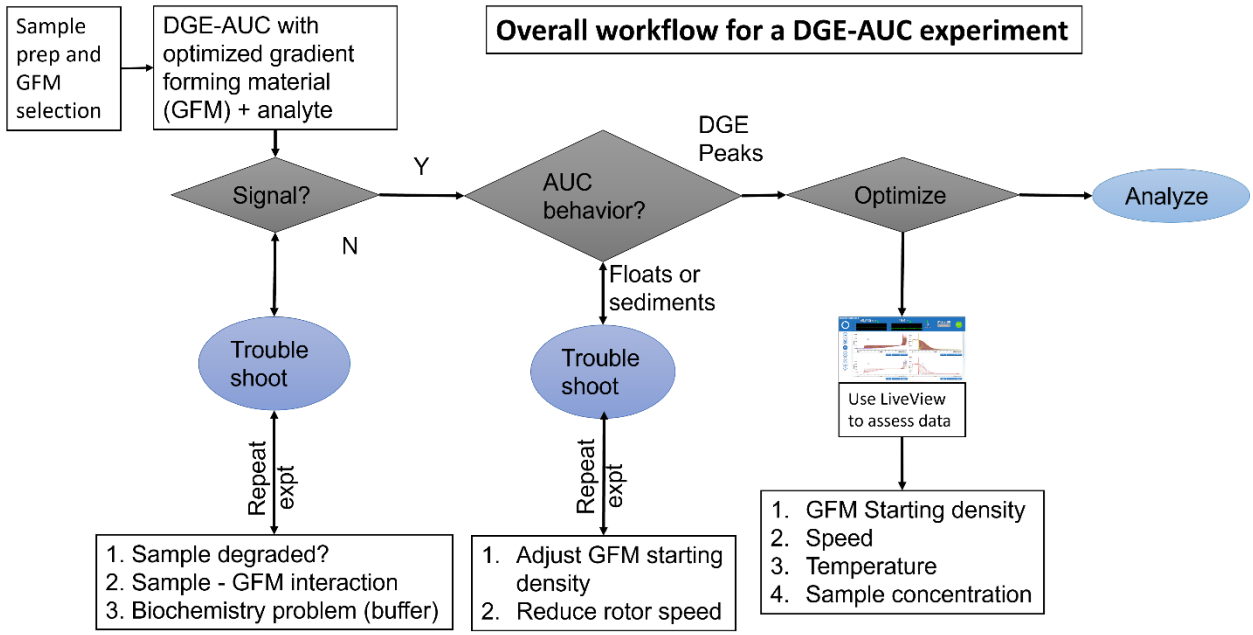

Figure 1: Workflow for a DGE-AUC experiment

The workflow for sample preparation is outlined in Figure 2. Sample preparation must be completed before starting the DGE-AUC workflow as shown in Figure 1. DGE-AUC samples have three components: the target analyte, the buffer and the gradient forming material (GFM). There are several biophysical and biochemical considerations which must be taken into account while preparing samples. These are shown in Figure 2 and include:

1. Target analyte must be at sufficient concentration to provide detectable signal and low noise, while not saturating the absorbance detector.
2. Target analyte must be stable for the experiment duration (~ 2 days).
3. Buffer and GFM must be chemically compatible with target analyte.
4. Buffer and GFM should not interfere with detection of target analyte using UV-Vis absorbance.
5. GFM should have a sufficiently high refractive index such that the density gradient curves are measurable by the Rayleigh Interference Fringe (RIF) detector.

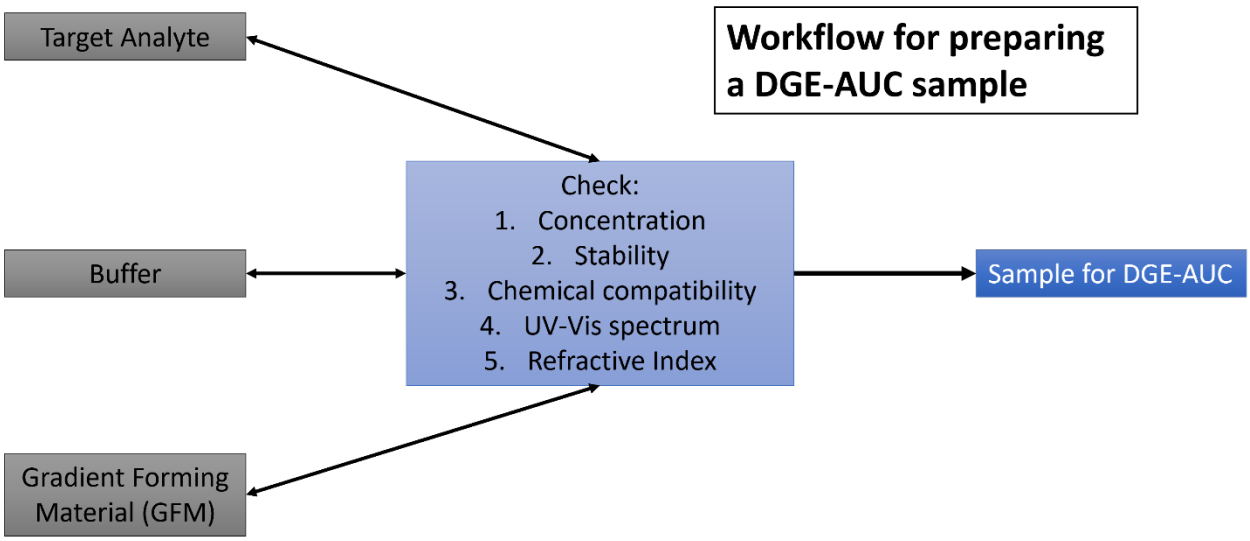

Figure 2: Workflow for preparing a DGE-AUC sample

Preparation of stock GFM solutions

The target parameter for stock GFM solutions is usually the final density in g/mL (or kg/L). GFMs like CsCl, $CsSO_4$, KBr, Sucrose, etc., are all available as solid powders. Therefore, it is necessary to prepare stock solutions of these GFMs in the relevant buffers. Now while these stock solutions are prepared to a target density, in practice, they are usually prepared by measuring out a known weight of solid chemical power and dissolving in a known mass of water-based buffer. Thus, stock GFM solutions are prepared at known concentrations, which must be converted to densities. This requires a conversion table, usually from data on density-concentration plots publicly available for various commonly used GFMs[3]. However, whenever possible, measure the density of the final GFM stock solution using a densitometer.

Preparation of high-concentration stock GFM solutions comes with the following caveats:

1. Stay below the relevant saturation point.
2. Storage at low temperatures can trigger precipitation.

Sample preparation equations

The equations governing sample preparation can be derived simply as shown below. We start by defining the relevant parameters in Table 1.

Symbol	Parameter	Comment	Example	Units
v_t	Total target expt vol	fixed	1000	µL
v_g	GFM stock vol	calculate		µL
v_b	Buffer + analyte vol	calculate		µL
d_t	Target sample density	variable, user-defined		g/mL
d_g	GFM stock density	fixed	1.7	g/mL
d_b	Buffer + analyte density	fixed	1.09	g/mL

Table 1: Defining the parameters for density and dilution calculations

It is important to note that the usual experimental protocol involves knowledge of the target GFM density and target experiment volume. Therefore, the dilution calculations are set up to provide the user with the volume of buffer (including analyte) and volume of GFM stock, which add up to the target experiment volume. This is shown below in Table 2.

From volume conservation	$v_t = v_g + v_b$ -- Eq(1)
From mass conservation	$v_t*d_t = v_g*d_g + v_b*d_b$ -- Eq(2)
Using Eq(1) to solve for v_b	**$v_b = v_t*(d_g - d_t)/(d_g - d_b)$ -- Eq(3)**

Table 2: Derivation of the dilution equation

Example calculations are shown below for preparing a dilution series of CsCl. The target density varies from 1.2 to 1.5 g/mL of CsCl, while the stock density of CsCl is set to 1.7 g/mL (see Table 1). The target experiment volume is 1000 µL in all cases.

d_t (g/mL)	v_b (µL)	v_g (µL)
1.2	819.67	180.33
1.25	737.70	262.30
1.30	655.74	344.26
1.35	573.77	426.23
1.40	491.80	508.20
1.45	409.84	590.16
1.50	327.87	672.13

Table 3: Examples of buffer volume and stock GFM volume to make up 1000 µL of experiment volume at different target starting densities of GFM

Characterizing UV-Vis absorption of the GFM and analyte

In the previous installment of this technical series (Demystifying DGE-AUC II: On gradient forming materials), we have already discussed the importance of characterizing the optical behavior as part of the selection criteria for GFMs. An example of this is provided for Sucrose in Figure 3. It can be seen that at concentrations below 30% w/w, Sucrose absorbs minimally at our wavelengths of interest (usually 230, 260, 280 nm), but displays a high enough refractive index (~1.38), that we expect a high fringe shift in the AUC RIF detector system.

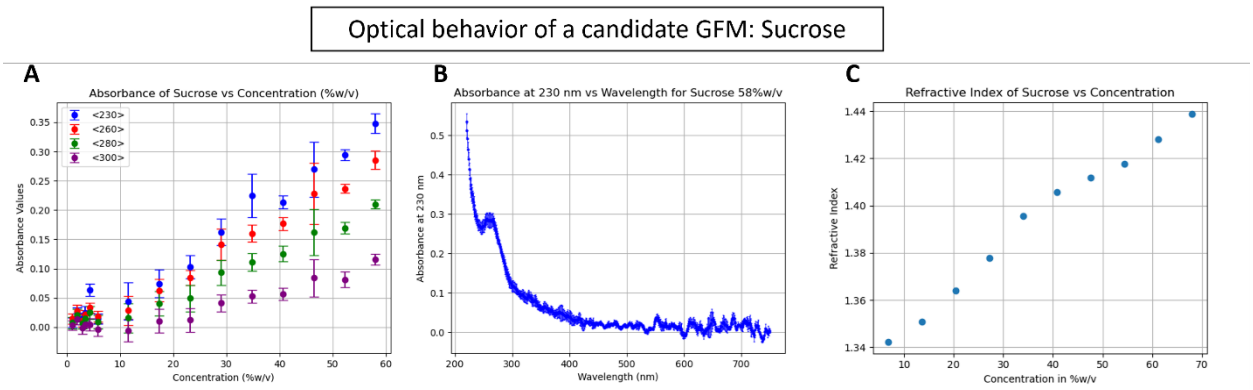

Figure 3: Optical behavior of sucrose; 3A: Absorbance (10 mm path) of sucrose at different wavelengths vs concentration; 3B: Full spectrum of sucrose at 58% w/v, and 3C: Refractive Index of sucrose vs concentration

On the other hand, Iodixanol absorbs very strongly at low UV wavelengths, as seen in Figure 4. This is also true for high concentrations of KBr, as seen in Figure 4. Thus, neither Iodixanol nor KBr are suitable as candidate GFMs for DGE-AUC experiments. This stands in contrast to the UV-Vis spectrum for CsCl, which shows negligible absorption throughout the full spectrum range. All UV-Vis data shown in this section was recorded on a Thermo Fisher Scientific NanoDrop 2000 or NanoDrop One spectrophotometer using 10 mm path and PBS blank. Note that vertical axes scales vary between samples. The Iodixanol spectrum was recorded from 190 nm to 850 nm, while CsCl and KBr spectrum were recorded from 220 to 850 nm.

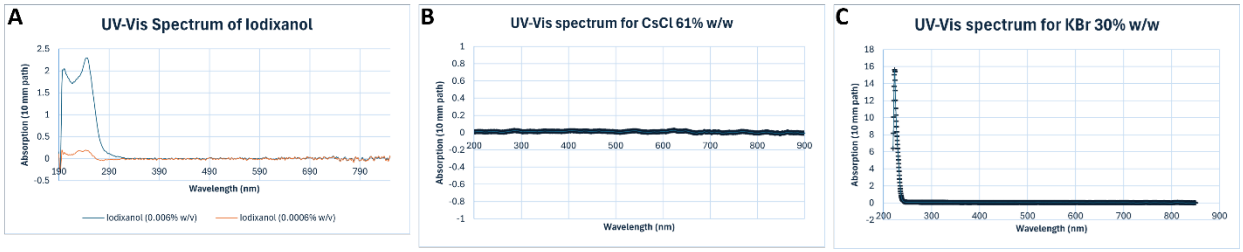

Figure 4: Absorption spectrum of (4A) Iodixanol; (4B) CsCl 61% w/w, and (4C) KBr 30% w/w in water

The refractive indices of CsCl and KBr at various concentrations is shown in Figure 5. Note that the Y-axes (RI) are scaled differently for the two GFMs. The samples are the same as used for the UV-Vis absorption vs concentration curve shown above. All the refractive index measurements performed in this study were done using a Reichert AR200 handheld digital refractometer. Measurements were made in triplicate against a water blank.

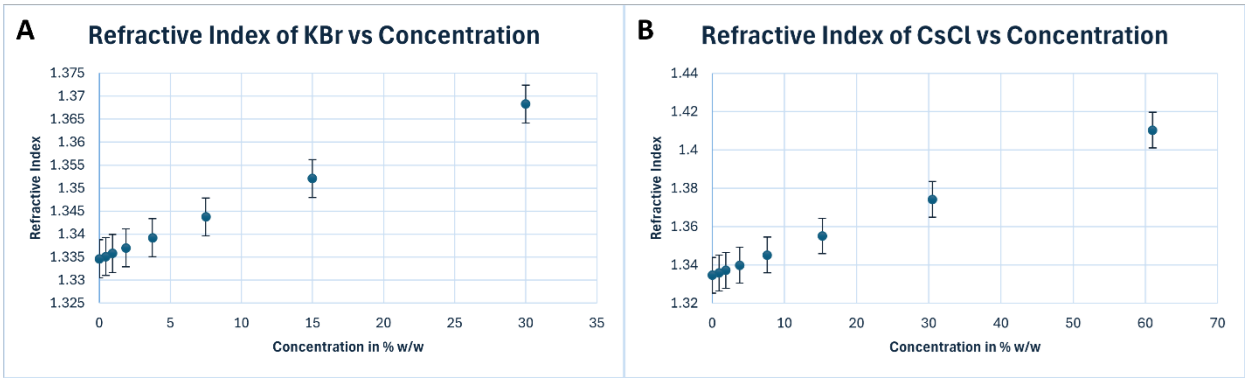

Figure 5: Refractive Indices of KBr and CsCl as a function of GFM concentration; 5A: KBr; 5B: CsCl

Thus far, we have discussed gradient forming materials and density gradients We will now focus on the analyte being characterized.

The first step is to measure the UV-Vis absorption spectrum of the analyte. The example in Figure 6 shows a UV-Vis absorption spectrum of BSA protein in PBS buffer recorded at two different concentrations. The spectra were recorded on a Thermo Fisher Scientific NanoDrop One instrument against a PBS blank buffer in triplicate. Error bars are shown. In this experiment, the spectrum is recorded from 220 to 850 nm. It is apparent that this sample has two absorption regimes which can be accessed for detection. The first is the low-UV region from 220 nm to about 240 nm. The second is the region from 275 to 285 nm. Both regions correspond to different biochemical moieties that will be discussed in the following section.

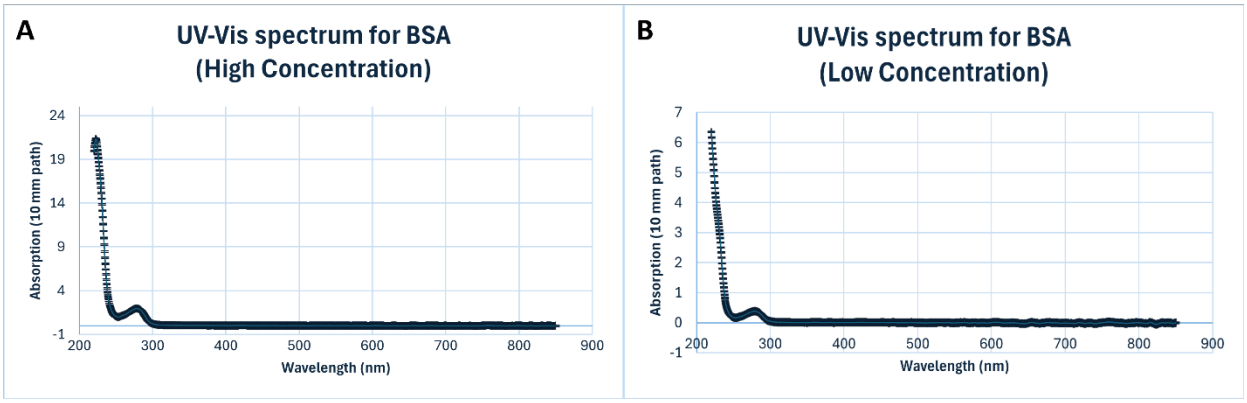

Figure 6: UV-Vis Absorption spectrum of BSA in PBS

The example shown in Figure 7 is for samples containing lipid nanoparticles or LNPs. The spectra for both Control LNPs and LNPs containing mRNA-GFP do not show a distinctive absorption maxima, but rather a gradual decrease of absorption from the low UV region to increasing wavelengths. This is indicative of scattering, rather than pure absorption – but the effect is the same in terms of reduced light intensity at the spectrophotometer detector. Absorption for the LNPs containing mRNA show a more gradual tapering off as wavelength increases. This may be due to the presence of mRNA cargo. Note that the spectrum for the mixture of both types of LNPs is shown in panel C with a different Y-axis scale.

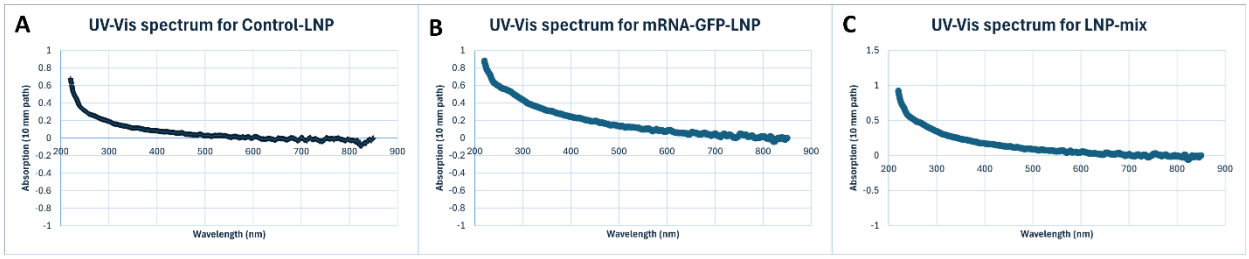

Figure 7: UV-Vis Absorption spectrum of LNPs in PBS; A: Control LNPs (diluted 5x), B: LNPs containing mRNA-GFP (diluted 5x), C: Mixture of Control LNPs and LNPs containing mRNA-GFP (both diluted 10x)

In the final example shown in Figure 8, we have measured the spectra of Doxoves (Doxorubicin loaded liposomes) and control liposomes. Doxoves (blue trace) have a prominent absorption peak at 490 nm, which is attributable to the drug Doxorubicin itself. The control liposomes (red trace) do not display the same absorption peak. Both traces have significant absorption at 230 nm, which also corresponds to a high output peak in the AUC absorption detector's flash lamp. This suggests that we can perform a multiwavelength experiment using 230 and 490 nm, wherein both liposomes will absorb at the lower wavelength, while only drug loaded Doxoves will absorb at the higher wavelength.

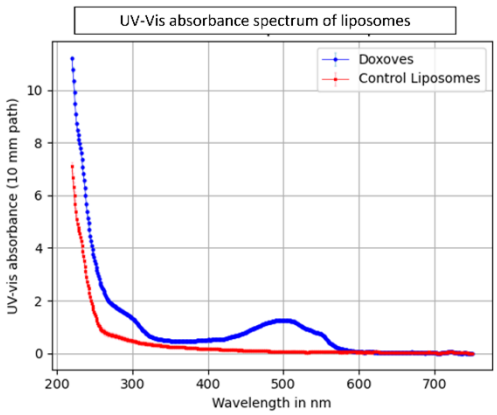

Figure 8: UV-Vis spectra of liposomes (Doxoves, i.e., Doxorubicin loaded liposomes in blue trace and control liposomes in red trace)

Choosing detection wavelengths

In general, it is necessary to pick a detection wavelength that corresponds to the biochemical moiety under investigation. For instance, while working with protein samples, 280 nm is the obvious choice because of aromatic amino acid sidechain absorption. However, if dyes can be chemically conjugated, or otherwise engineered into the system of interest (such as a GFP domain), then detection wavelengths can be chosen which allow a higher degree of selectivity. This could include examples like a protein conjugated to Alexa Fluor 488[4] dye, mixed with other non-conjugated proteins. The protein of interest is detected via absorption at 488 nm without picking up signal from the other proteins. The following table shows a representative list of absorption wavelengths and their corresponding biochemical moieties.

It is necessary to compare the spectra thus obtained with the spectra of the GFM. In the case study with liposomes, the chosen GFM is sucrose, for which the optical properties are shown in Fig 3. Thus, we know by comparison that 230 and 490 nm do not represent any GFM-related absorption peaks – and can be safely used to exclusively monitor the analytes. This case study will be discussed in a subsequent installment of this technical series.

It can also be seen that CsCl is suitable across a wide wavelength range, as seen in Figure 4. However, KBr and Iodixanol are not suitable at low UV. This means that KBr and Iodixanol may not be appropriate GFMs for DGE-AUC experiments with LNPs and liposomes, where detection is usually at 230 nm.

Wavelength (nm)	Biochemical Moiety
230	Peptide bonds and Mie scattering
260	Nucleobase rings
280	Aromatic amino acid sidechains
340	Intrinsic Tryptophan
490	Fluorescein, GFP, other dyes

Table 4: Absorption wavelengths and the corresponding biochemical moieties

Finally, Table 5 shows the refractive indices (RI) as well as absorption at 280 nm for a few different samples of interest. These samples were all in PBS buffer. Both RI and absorption measurements were made using PBS blank in triplicate. Standard deviations of readings are also provided. All of these samples have very low RI readings and are hence not expected to generate significant RIF readings in the Optima AUC instrument, as compared to the GFMs.

Sample	Average (Refractive Index)	Stdev (Refractive Index)	Average (Abs280)	Stdev (Abs280)
BSA Hi Conc	1.33E+00	0.00E+00	1.89E+00	2.83E-02
BSA Low Conc	1.33E+00	0.00E+00	3.70E-01	8.16E-03
Control-LNP	1.34E+00	8.16E-05	2.27E-01	2.38E-02
mRNA-GFP-LNP	1.34E+00	8.16E-05	5.07E-01	8.56E-02
LNP-mix	1.34E+00	9.43E-05	4.11E-01	2.82E-02

Table 5: Optical measurements on a few biological samples

Setting up a DGE-experiment

The steps involved in setting up a typical DGE-AUC experiment are shown below. These steps are representative of the case studies which will be discussed later in the series.

1. Load sample(s) and reference(s) into AUC cells (using 2-sector centerpieces). Sample load volume should be ~ 420 to 450 µL. Reference load volume should be slightly greater than sample load volume. This ensures that the sample meniscus is below the reference meniscus in terms of radial position, and leads to easier visualization and analysis.

2. Weigh AUC cells and load the AUC rotor with balanced AUC cells in opposite positions.

Experimental (data acquisition) parameters

The parameters used for a typical first-pass DGE-AUC experiment are shown in Table 6. Note that the Rayleigh Interference Fringe (RIF) detector is tasked to collect twice as many scans as a single wavelength in the absorbance detector, but with half the time-gap. This is intentional – it allows us to monitor the approach of GFM equilibrium closely and is facilitated by the faster read time of the RIF detector.

Expt Stage	Rotor speed (krpm)	Delay (sec)	Detector	Wavelengths (nm)	Time between scans (sec)	Total # of scans	# of cells	Total duration (hr)
1	40	0	Abs	230, 490	600	300	7	50
			RIF		300	600	7	50

Table 6: Typical data acquisition parameters for a DGE-AUC experiment

Temperature Equilibrium

Usually, Sedimentation Velocity experiments (SV-AUC) require a temperature equilibrium stage of 2 hrs. This prevents convection in the AUC cell which can distort SV-AUC data. This step is optional since DGE-AUC experiments usually extend for 12 hours or longer, and only the final equilibrium data scans are used for analysis. Thus, a DGE-AUC experiment, by its very nature, allows for thermal equilibrium to be established simultaneously with the transport equilibrium processes. This is beneficial in terms of time saved.

Conclusions

In this installment of this multi-part technical series, we discussed how to prepare samples for DGE-AUC experiments. In particular, we have looked at the optical properties of both gradient forming materials and target analytes in detail and discussed the nuances of selecting a GFM that is optically compatible with the target analyte. In addition, we touched upon the possibility of using multiwavelength absorption detection to selectively track the movement of different biochemical species in the sample. Finally, we discussed the steps involved in setting up a DGE-AUC experiment and provided a sample parameter set for a DGE-AUC experiment. In subsequent installments of this series, we will discuss data analysis, and view case studies with different sample types.

References

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