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AUCINSIGHTS

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Sample concentration in the Analytical Ultracentrifuge AUC and the relevance of AUC data for the mass of complexes, aggregation content and association constants

INTRODUCTION

Biological Macromolecules can be present as monomers but often build complexes with specific functions that are important and/or essential for biological systems. However complexes of biological macromolecules can also form as an unwanted result from conditions during the preparation or isolation or storage conditions.

While we typically speak about associations if it comes to natural complexes we typically speak about aggregation and aggregates when we describe unwanted complexes. Despite their different origin both types of complexes can have similar characteristics, for example they can be either reversible or irreversible. Aggregates can result in very large complexes, even be in a size where they become visible to the naked eye – but this is not always the case. Aggregation can also result in aggregates of the size of oligomers and even a Dimer can be a result of an aggregation. Thus both associations and aggregates can be of the size of a multimer and so be in the same size range.

Also for both type of complexes parameters such as the environment of the molecules (e.g. buffer composition, ionic strength, pH etc.) and the sample concentration can have a significant impact on the size of the complexes built or the amount of the molecules that are organize in complexes. The organizing in complexes, e.g. is a self-association of proteins can be very concentration dependent *1.

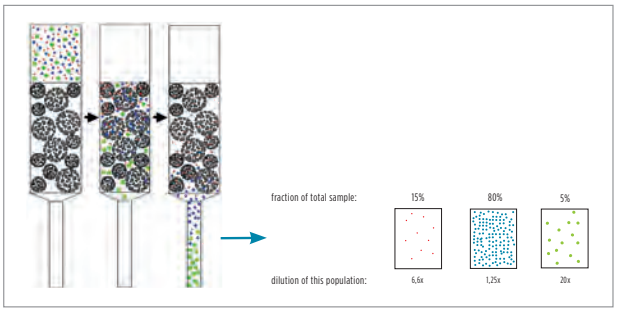
That makes them challenge for a characterization technique. The best case for a characterization technique for systems that have or might have a concentration depending interaction and/or includes complexes that are complexed by weak interactions should be able to

a. cope with a heterogenous sample and
b. characterize the sample at the concentration of interest or at least let the scientist control effect through changes of the sample concentration

EXAMPLE 1

Long-standing industry standard for measuring levels of protein aggregates

As one example for a characterization technique we first look at the long-standing industry standard for measuring levels of protein aggregates which is Size Exclusion Chromatography, SEC, *2. With this technique different populations of the sample are separated on a column, -the separation based on different permeation of the analytes into a porous matrix material with controlled pore sizes- and eluted separately. The elution time then is taken as a measure for the size of the molecules and complexes in the different populations. This size is either determined by a comparison to a size standard measured with the same configuration or by connecting the SEC to a detector that is directly measuring the mass. This technique can cope with a heterogenous sample, but regarding the sample concentration this technique has s specific characteristic: Besides a general dilution through diffusion on the column an additional dilution results from the separation of different populations. Generally the smaller the fraction of a population, the higher it´s dilution. The reason is that each population is eluted in the volume that is the loading volume plus the increase of this volume by diffusion on the column. The smaller the fraction of a specific population, the higher the dilution.



schematic overview on a 3 population sample separated by SEC. In this example fractions of the populations and dilutions that result are solely from the separation for each of the populations

As a result of the separation (there is additional dilution through diffusion) each population gets diluted by the factor of it´s fraction, see below for the example of a main population of 80% medium size particles, a population of 15% smaller particles and 5% aggregation content.

*1: There are more things to be considered for SEC like e.g. interaction between sample and the matrix (surfaces of the column material) , a filtration in the frit above the column material linked to SEC. These topics are not discussed here, for such refer to e.g. reference *2)*

EXAMPLE 2

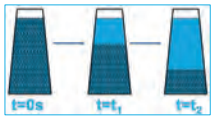
Now, How does Analytical Ultracentrifugation handle sample concentration?

Two types of experiments are typically performed with AUC: Sedimentation Velocity (AUC-SV) and Sedimentation-Diffusion Equilibrium (AUC-EQ).

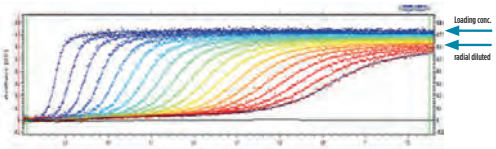
AUC-SV: Sedimentation Velocity is performed at high speed, the centrifugal acceleration depletes particles away from the center of the rotor and creates a pellet at the bottom of the cell. During this process a boundary between the sample and depleted buffer moves from a smaller to a larger radius, the speed and shape of this boundary containing information about the mass, size & shape of the particles.

The concentration of the sample below that boundary stays at the loading concentration except that the radial shape of the compartment results in a dilution of approx. 10-max. 20% as a specific volume of the sample moves into a slightly larger volume when moving to larger radius positions *6.

The boundary itself has is s-shaped as a result of a. diffusion and b. sample heterogeneity. The speed of the midpoint of this boundary represents the sample at a concentration between loading concentration and radial diluted sample. and if the radial dilution affects the sample, this would change speed over the experiment, which would be recognized during data analysis. All data here are fitted using thermodynamics, thus the scientist has full control over all effects through dilution.

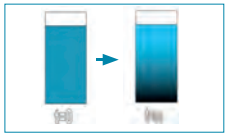


AUC-SV – particles are moved towards the pellet position, above a boundary depleted buffer is present are sedimented

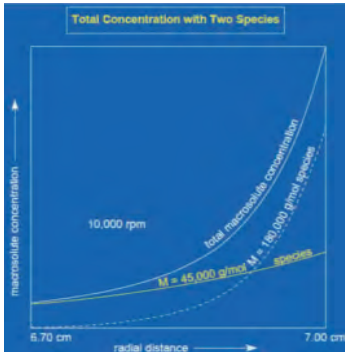


a series of scans of a AUC-SV experiment (not all scans showed), arrows at the right show level plateau region for early (loading conc.) and for late scans (radial diluted)

AUC-EQ: Sedimentation-Diffusion experiments are performed at lower speeds so does not create a pellet at the bottom of the cell. Simultanous transports through sedimentation and diffusion produce a gradient across the sample compartment and an equilibrium distribution is reached when the opposing transports reach a balance. The equilibrium distribution typically ranges from concentration 0 to high concentration - in each single profile. The equilibrium distribution can thermodynamically be described by a superposition of Boltzmann functions, experimental data are fit with least squares methods. Results an include the Molecular weight, Aggregation states and thermodynamic parameters like e.g. the association constants. This enables the characterization of an association including association constants for a full range of concentrations. Again -as thermodynamics is being used for the data analysis - the scientist has full control over concentration effects.



AUC-EQ – at lower radial centrifugal force, rcf, the transports through Sedimentation and Diffusion come result in a concentration profile in the sample



typical concentration profile resulting from an AUC-SV experiment (total macrosolute concentration), exponential profiles below represent two different species as calculated form the measured profile

SUMMARY

In both typically used experiment types of AUC, AUC-SV and AUC-EQ the scientist can control the influence of dilution on the particle populations. While in AUC-SV the sample is slightly diluted through radial dilution (approx.. 10-20%) and the boundary region a steep concentration gradient is produced in AUC-EQ experiments. Latter is wanted as it allows for the characterization of an association at many different concentrations.

The fact that thermodynamics can be used for the analysis of AUC data – which is why Analytical Ultracentrifugation is also called a first-principle technique – enables the scientist to see and analyze concentration effects on the sample at all times. The parameters calculated with this first-principle technique do not require a standard. As AUC analyzes the molecules and complexes while they float free there is no risk of any matrix effects.

OPTIMA AUC

- First-principle technique that does not depend on a matrix and does not require standards
- Samples are analyzed in their native state with almost no buffer restrictions
- One experiment reveals information about shape, diameter, mass, stoichiometry, purity, formulation heterogeneity, aggregation, association and conformation of a protein or protein complex
- Optical systems:
  - Rayleigh Interference
  - UV absorbance
- Sample volume:
  max. volume for 2-sector centerpieces: 450 µl
  max. volume for 6-channel equilibrium centerpieces: 120 µl
- Wavelength range: 190 – 800 nm
- Molecular weight range:
  10^2 Da (i.e. Peptides/Oligosaccharides)
  - 10^8 Da (i.e. Viruses/Organelles)
- Concentration range:
  UV absorption:
  0.005 – 1-2 mg/ml Lutenizing Hormone
  Interference:
  0.025 - 4-5 mg/ml BSA

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