LABORATORY OF

ELEMENTARY BIOPHYSICS

Experimental exercises for III year of the First cycle studies
Field: "Applications of physics in biology and medicine"
Specialization: "Molecular Biophysics"

PPB15d

Determination of sedimentation coefficient for mutant of Green Fluorescent Protein by analytical ultracentrifugation method with fluorescence detection

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Introduction

Ultracentrifugation accelerates the sedimentation process, and helps in the separation of molecules with different masses. In modern ultracentrifuges the sample rotate at speeds up to 60 000 rpm, what corresponds to acceleration 290 000 g. Under such conditions, the protein molecules achieve the weight of even a few newtons, and move away from the axis of rotation.

The use of ultracentrifugation as a research method begins in the 20s of the twentieth century, when Theodor Svedberg constructed a centrifuge with an optical detection system and Ole Lamm presented a mathematical equation that describes the phenomenon of sedimentation in the field of centrifugal force. Nowadays ultrafiltration is used in scientific and industrial laboratories. It enables the analyze of sample heterogeneity, weight and conformation, association (including autoassociation and the stoichiometry of association) for the molecules with weight from a few hundred to tens of millions of daltons. The advantages of ultracentrifugation are: lack of artifacts related to the interaction of macromolecules with the matrix, no need to use the standards (which gives the possibility to compare data obtained over the years) and working in aqueous solutions, what allows to have the conditions close to physiological.

Physical basis of the method

There are three forces that affect the rotating molecule:
centrifugal force F, buoyant force W and friction force T (Fig. 1):

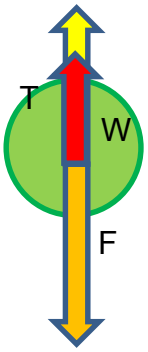

Figure 1. The forces acting on a molecule during a centrifugation

Centrifugal force is described by the expression:

$$F = ma = V\rho a = V\rho\omega^2 r$$

where:
m - mass of the molecule,
ω - angular speed,
r - distance from the axis of rotation,
a - centrifugal acceleration,
V - volume of the particle,
ρ - density of the particles.
buoyant force is described by the expression:

$$W = m_s a = V\rho_s a = V\rho_s \omega^2 r$$

where

ρ_s - density of the liquid

dynamic friction is described by the expression:

$$T = f(dr/dt)$$

where:

f - friction coefficient, depending on the properties of the molecule and the viscosity of the solution

For the spherical shape of the particles and laminar flow of the liquid Stokes formula can be used:

$$T = 6\pi\eta R(dr/dt)$$

where:

R - radius of macromolecules,

η – liquid viscosity.

After a short time from the start of spinning the forces acting of the particles equilibrating and the molecules begin to move with a constant velocity

$$F + W + T = 0$$

Hence:

$$F - W = V(\rho - \rho_s)\omega^2 r = f\frac{dr}{dt}$$

When analyzing movement of particles in the centrifugal force field the sedimentation coefficient is introduced. It is equal to the velocity of the molecule per unit of centrifugal acceleration. The unit of sedimentation coefficient is 1 S (Svedberg) = 10^{-13} s.

$$S = \frac{1}{\omega^2 r}\frac{dr}{dt}$$

When we determine the value of the sedimentation coefficients for the macromolecules suspended in a solution, we can get information about the structure and behavior of these particles in the environment close to natural.

Determination of the sedimentation coefficient

From the definition of sedimentation coefficient, after separation of variables and integration, the following relationship is obtained :

$$\ln r = S\omega^2 t + const$$

where r – location of the center of the border (see below).

From these equation, plotting the ln(r) as a function of time and calculating the value of ω^2 from experimental data we obtain the S-value.

Cuvettes

Ultracentrifugation cuvettes are used under enormous congestion, and therefore have a special design. They consist of several elements (Fig. 2). The central part, where the test samples are placed is closed with quartz or sapphire windows and surrounded by guards, holders and gaskets, designed to protect the cuvette and prevent it from leaking. This construction is placed in a titanium or aluminum housing, which is twisted in a special vice.

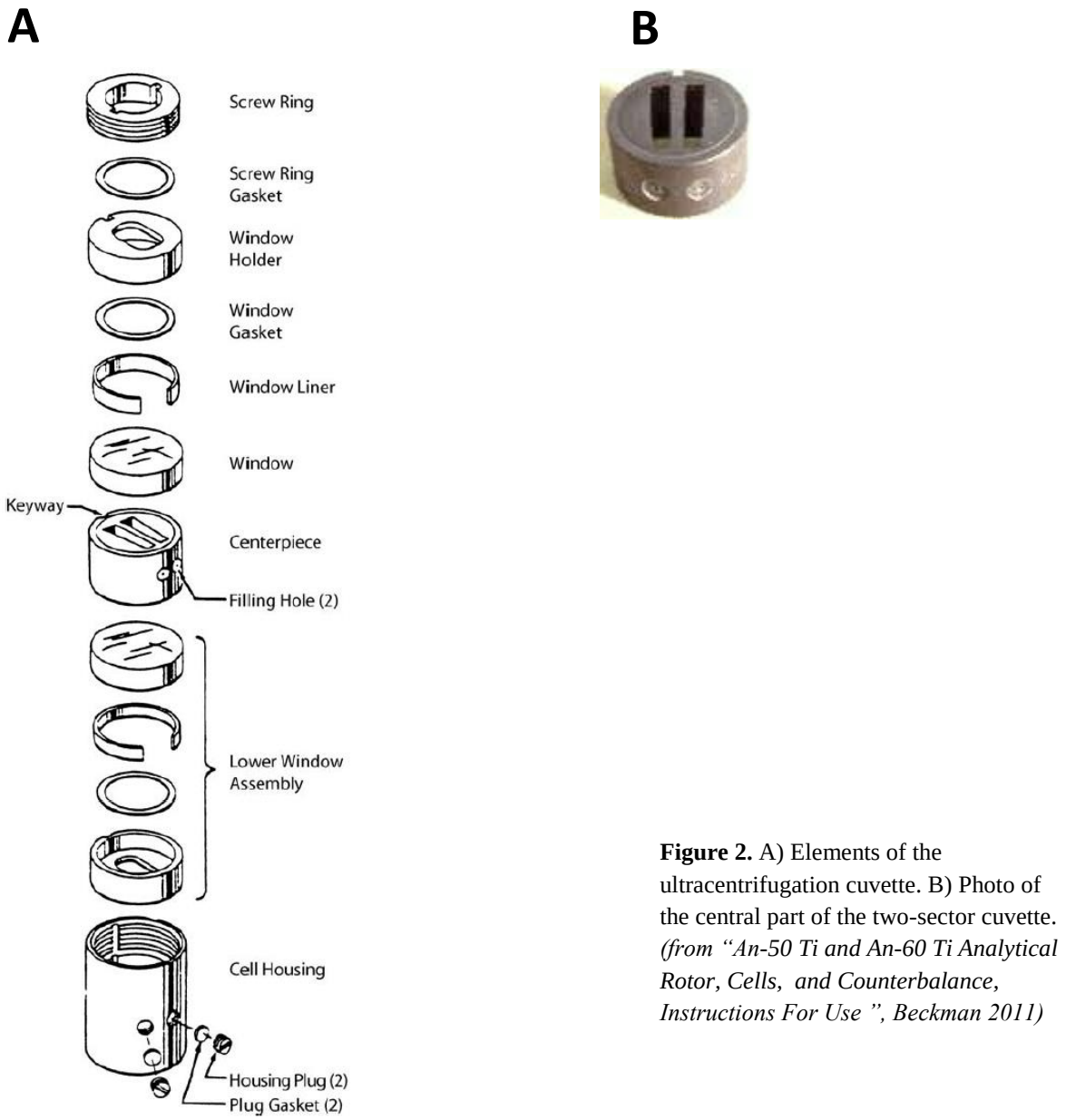

Figure 2. A) Elements of the ultracentrifugation cuvette. B) Photo of the central part of the two-sector cuvette. *(from "An-50 Ti and An-60 Ti Analytical Rotor, Cells, and Counterbalance, Instructions For Use ", Beckman 2011)*

Twisted cuvette is filled with a solution (in the case of two-sector cell one of the sectors may include a sample and the second a reference buffer or, in the case of fluorescence detection, both of sectors can contain the samples) and placed in a titanium rotor together with the calibration cuvette, different for absorption-interference and for fluorescence detection.

Observation methods of the sedimentation process

There are three detection methods of the phenomena, taking place in the cuvette: absorption, interference and fluorescence. The particular choice of the method is related to the properties of the molecules. If molecules (proteins, nucleic acids), absorb radiation, the most appropriate method is to measure the absorption. If they have emission properties (green fluorescent protein

or labeled macromolecules) the proper methods is based on fluorescence measurements. In the case of the macromolecules not having the ability to absorb or emit light (polysaccharides and hydrocarbons) we have to measure the scattering.

Interference detection

In the interference detection system the light beam is delimited between two parallel slots and passing through the two cells of cuvette containing the sample and buffer. The speed of light when passing through the higher density solution decreases. If the density of sample is greater than the density of reference buffer, the wave passing through the sample is delayed relatively to the wave passing through a buffer. One can observe a shifting of the interference fringes due to the difference in density of the sample and the reference (Fig. 3).

Figure 3. Shift of the interference fringes occurring during ultracentrifugation

Absorption detection

The detection system is based on the phenomenon of radiation absorption by a molecule having absorption properties.

A xenon lamp is the light source. We observe the absorption of light by the sample cell containing absorbing molecules in comparison to a reference cell containing the buffer. You can select the wavelength of observation in the range 190 - 800 nm. For the complex samples it is possible to monitor several components through the observation of the absorption at different wavelengths.

Fluorescence detection

The fluorescence detection (Fluorescence Detector System FDS) is equipped with a laser which emits light at 488 nm, a lens system and a dichroic mirror that reflects the laser radiation towards the sample, but transmit the radiation emitted by the sample towards the photomultiplier. Special cut filter allows you to collect the emitted radiation in the range of 505 - 565 nm (Fig. 4). Since the excitation wave is 488 nm the sample must absorb this wavelength and emit radiation in the range 505 - 565 nm. In this region (505 - 565 nm) absorption must be low in order to avoid the inner filter effect. The ideal biological marker is GFP or EGFP. They absorb the radiation in

the proper range (absorption maximum at 489 nm) and emit above 505 nm. Samples that aren't fluorescent are labeled using specific fluorescent markers such as fluorescein or Alexa488. Fluorescence detection is highly sensitive, allows for the observation of fluorescent samples with the concentrations of the marker in the range of pg/ml - ng/ml, whereas the absorption detection is suitable for a range of μg/ml - mg/ml, depending on the molecular extinction coefficient, and interference detection is suitable for the concentration of the sample at μg/ml.

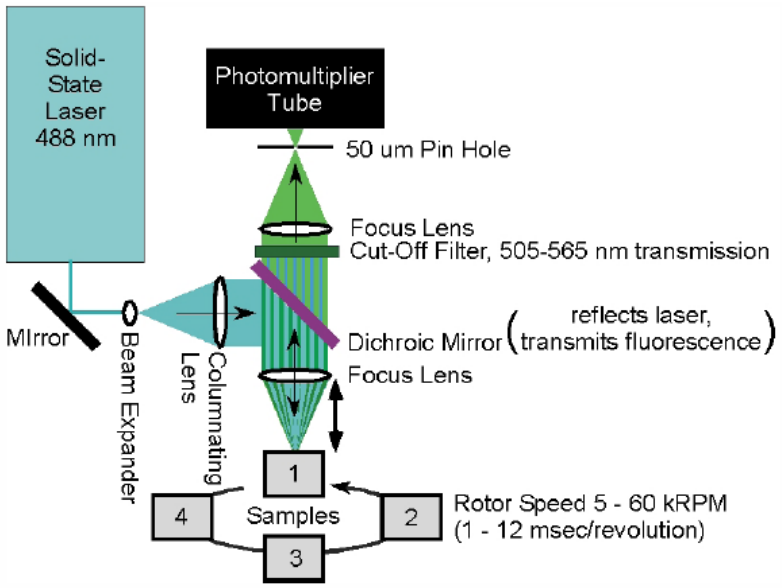

Figure 4. Fluorescence detector *(Aviv).*

Green fluorescent protein (GFP) and a mutant with enhanced fluorescence (EGFP)

GFP is a small protein composed of 238 amino acids, mainly beta-sheet, arranged in the beta-barrel (Fig. 5). Interior of the beta-barrel is well protected from the environment. Under these conditions, three adjacent amino acids: Gly67 Ser65 and Tyr66 form a chromophore responsible for the emission of protein. Chromophore excited in the range from ultraviolet to blue, emits intensive green radiation. The appearance of emission does not require additional biochemical reactions and interactions with other molecules. This property allows for the use of GFP as a neutral, intracellular marker. If the gene of interest, fused with the GFP gene, is introduced into the cells, it is easy to find the location of the expressed protein-GFP construct, observe what is happening to it (does it multiply or move?).

Due to the widespread use of GFP as a biological marker many mutants, with altered fluorescent properties, were constructed. In addition to mutations affecting the color of light emitted by

GFP, it is also possible to introduce the change which improves significantly the emission intensity.

This mutant is known as EGFP. The following mutations were made to it: S65T, which simplifies the absorption and excitation spectra and F65L – which improves the intensity of green fluorescence. EGFP shines several times brighter than GFP what makes it the more sensitive biological marker.

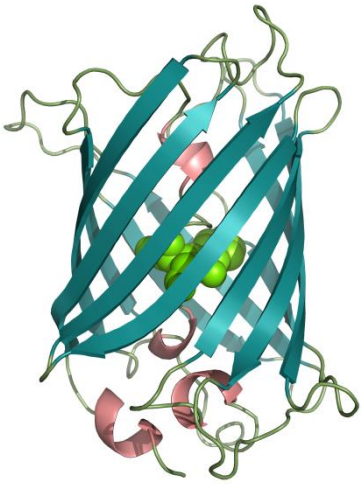

Figure 5. The green fluorescent protein (GFP). Inside the β-barrel the location of the fluorescent chromophore is marked.

Types of experiments performed using analytical ultracentrifugation

There are two types of ultracentrifugation experiments:

1) Sedimentation velocity (SV)

2) Sedimentation equilibrium (SE)

Sedimentation velocity

Sedimentation velocity experiment can be performed at high speed of centrifugation (40 000 - 60 000 rpm). The experiment lasts a few hours. We can see that the boundary between the clear liquid and macromolecules suspension moves during experiment (Fig. 6). Thus, we can determine the sedimentation and diffusion coefficients and next, calculate what is the molar mass of the suspended particles.

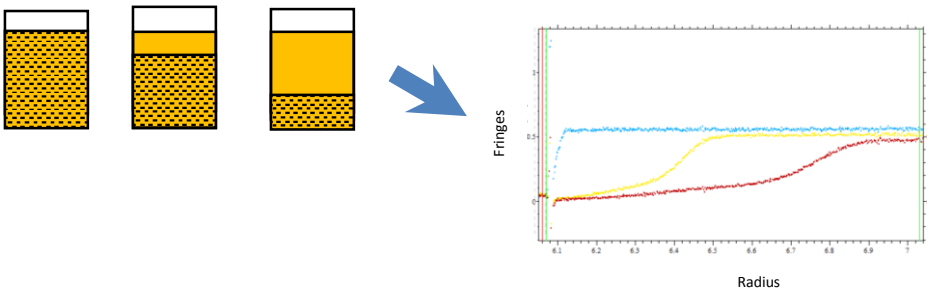

Figure 6. Sedimentation velocity. Shifting of the boundary between the clear solution and the suspension and the corresponding image on the monitor screen.

Sedimentation equilibrium

Sedimentation equilibrium experiment runs at much lower speeds (3000 - 15 000 rpm). After a long time, sometimes a few days, equilibrium between the centrifugal force and the diffusion force is established, distribution of molecules in the cell is determined, their concentration increases continuously with distance from the axis of rotation and there is no clear boundary between the clear liquid and suspension (Fig. 7). Analysis of the concentration distribution of dissolved molecules can determine the composition of the mixture and the weight of the components.

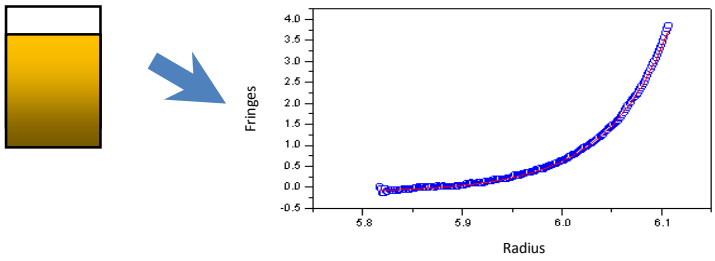

Figure 7. Sedimentation equilibrium experiment. During the experiment the gradient of macromolecules concentration in the cuvette (left side) is determining and the corresponding image on the monitor screen (right side) is seen.

The aim of the exercise is:

• know the principles of operation and maintenance of analytical ultracentrifuge

• know the unique method of fluorescence detection

• carry out the sedimentation velocity experiment involving observations of moving fluorescent biomolecules suspension by centrifugal force

• determination of the sedimentation coefficient

• discussion of the results and its comparison with the literature data

Entry requirements

Prior to the experimental part, students must pass an preliminary test. The teacher decides about the form of this test. The material on the analytical ultracentrifugation, object of the investigations, and method of the experiment performance is presented in this instruction and placed in the bibliography.

Following topics may be discussed during the test:

1. Physical basis of analytical ultracentrifugation
2. Comparison of experimental methods (SV and SE). When are they used for? What information can we get through them?
3. Detection systems in analytical ultracentrifugation
4. Characteristics of GFP and EGFP. Why are they good labels in the case of fluorescence detection method?
5. Description of the most important elements of the ultracentrifuge. How to prepare the cell for measurement?
6. The method of determining the sedimentation constant

Realization of the experiment

In this experiment we will determine the sedimentation coefficient of EGFP.

We can use

• EGFP solution at a concentration of 40 μg/ml in 50 mM phosphate buffer pH 8.0 with 0.3 M NaCl

• analytical ultracentrifuge

• cuvette, which must be build

• fluorescein solution at a concentration of 100 μM

• a buffer 10 mM Tris/HCl pH 7.8 with 100 mM NaCl to prepare a dilute solution of fluorescein

The stages of the experiment:

• Preparation of the cuvettes

• Preparation of fluorescein solution at a concentration 1 μM in the buffer 10 mM Tris/HCl pH 7.8 with 100 mM NaCl

• Filling of the fluorescent calibration cuvette with fluorescein solution.

• Preparation of sample EGFP concentration of 1 μg/ml and placing it in both sectors of cuvette, then in the rotor and centrifuge

• Installing a fluorescence detector

• Start of the measurement, at first performing the calibration, selecting the proper voltage at the photomultiplier and gain

• Collection of the experimental data and stopping the centrifuge

• Determination of the sedimentation coefficient by the method described in the section "Determination of sedimentation coefficient "

• Comparison of the results with the literature data.

• Are the results consistent with the literature? What could be the cause of the discrepancy?

Note:

Due to the uniqueness of the apparatus all operations related to the preparation of the sample, and start of the measurement take place under the supervision of the assistant (some steps of the experiment are performed by the assistant).

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