Sedimentation
Electrophoresis
Mass spectrometry

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Sedimentation

In general, the aim is to describe the size or the mass of the particles.

The methods based on gravitation are effective only in the range of 2-50 µm particle radius.

*In the case of smaller molecules the sedimentation is based on the method of centrifugation*
Sedimentation in gravitational force

\[ F_{\text{upthr}} = \rho_0 Vg \]

\[ F_{fr} = fv \]

The difference of the gravitational and the upthrust force accelerates the particle, until they reach an equilibrium with the frictional force.

After that the speed of sedimentation is constant \((v = \text{const.})\):

\[ F_g - F_{\text{upthr}} = F_{\text{frictional}} \]

\[ \rho Vg - \rho_0 Vg = fv \]

\( \rho_0 \): density of the medium, \( m \): mass, \( \rho \): density, \( v \): speed of the moving sphaerical molecule.
\[
\rho V_g - \rho_0 V_g = fv
\]
\[
V = \frac{4}{3} r^3 \pi
\]
\[
F_{\text{frictional}} = 6\pi \eta_0 rv
\]

For sphaerical particles

Stokes’ law of friction

\(\eta_0\): viscosity of the medium,
\(v\): speed, \(r\): radius of particle

\[
(\rho - \rho_0)\frac{4}{3} r^3 \pi g = 6\pi \eta_0 rv
\]

\[
v_{\text{sed}} = \frac{2r^2 (\rho - \rho_0) g}{9\eta_0}
\]

can be measured

can be calculated
Sedimentation in centrifugal field:
1. Sedimentation velocity method

**Aim:** determination of molecular mass ($r \approx nm$)

**How to calculate forces?**

\[ F_{\text{frictional}} = f v \]

\[ F_{\text{centrifugal}} = m r \omega^2 \]

\[ F_{\text{upthrust}} = \rho_0 V g = \rho_0 \frac{m r \omega^2}{\rho} \]

"Archimedes": the weight of the excluded solvent

Centrifugal field: „accelerating co-ordinate system“
How long is the particle accelerating?

It can accelerate while: \( F_{\text{frictional}} = F_{\text{centrifugal}} - F_{\text{upthrust}} \)

\[
f v = m r \omega^2 - \rho_0 m \frac{r \omega^2}{\rho} = m r \omega^2 \left( 1 - \frac{\rho_0}{\rho} \right)
\]

Is then \( v = \text{const.} \)?

The value of the centrifugal field depends on the radius \( (a_c = r \omega^2) \), it increases further from the axis

\[
\downarrow
\]

In the case of sedimentation the velocity of the particle increases also further from the axis. (velocity is place-dependant)

No.
The Svedberg

Sedimentation coefficient

Unit: $1 \text{ Sv} = 10^{-13} \text{s}$

Sedimentation speed by unit field strength

$S = \frac{v}{r \omega^2} = \frac{m(1 - \frac{\rho_0}{\rho})}{f}$

Can be calculated

Can be measured

Shape factor!

There is a correlation between shape factor ($f$) and the diffusion constant ($D$):

$$f = \frac{kT}{D} = \frac{RT}{ND}$$

where $k$ is the Boltzmann constant, $R$ is the universal gas constant and $N$ is the Avogadro-number.

To determine the mass one needs to combine the sedimentation method with diffusion measurements.

Theodore Svedberg
Swedish chemist
(1884-1971)
Nobel-laureate in 1926
The process of sedimentation

particles

concentration

first derivative
How can we detect the molecules upon sedimentation?

Schlieren optical system

At the sedimenting meniscus concentration gradient equals with refraction index gradient. This can be transformed into a peak-shaped signal (derivation), detected by the Schlieren optical system and recorded on video.
The sedimenting meniscus

Comparison of the data obtained from the (a) schlieren, (b) interference, (c) photographic absorbance, and (d) photoelectric absorbance optical systems. ((a) (b) and (c) are taken from Schachman, 1959. Reprinted with the permission of Academic Press.)

2. Sedimentation equilibrium method

Particles are awaited to reach the bottom of the tube: their average sedimentation speed is 0.

A balance is set between the sedimentation and the thermal diffusion.

The energy of the thermal movement brings a portion of the particles into higher energy state.

Near the bottom of the tube a given distribution of the particles is present.
At \( r_1 \) and \( r_2 \) distances from the axis the ratio of \( n_1 \) and \( n_2 \) concentrations can be calculated from the Boltzmann-distribution:

\[
\frac{n_1}{n_2} = e^{-\frac{E_1 - E_2}{kT}}
\]

The difference of \( E_1 \) and \( E_2 \) potential energy:

\[
E_1 - E_2 = \frac{m}{2} \omega^2 \left( 1 - \frac{\rho_0}{\rho} \right) (r_2^2 - r_1^2)
\]
\[
\frac{n_1}{n_2} = e^{-\frac{E_1 - E_2}{kT}}
\]

Replace \((E_1 - E_2)\) and take the logarithm:

\[
\ln \frac{n_2}{n_1} = \frac{m \omega^2}{2kT} \left(1 - \frac{\rho_0}{\rho}\right) \left(r_2^2 - r_1^2\right)
\]

\text{can be measured} \quad \text{can be calculated}

\textit{The shape factor and additional diffusion measurements are not necessary.}
The density of the molecules (ρ) can be measured applying a density gradient centrifugation.

Remember: \[ F_{up} = \left( \frac{\rho_0}{\rho} \right) m r \omega^2 \quad \text{and} \quad F_c = m r \omega^2 \]

if \( \rho_0 = \rho \), then \( F_c = F_f \) and \( v = 0! \)

Can be obtained by centrifuging small particles which have large density (e.g. CsCl, CsBr)

**Centrifuging the sample in this medium particles will stop at their corresponding density.**
An example: Gradient centrifugation of infected Red Blood Cells

Infected cells are shown on the left, separated on a continuous gradient of increasing percoll concentrations. Different stages in the intraerythrocytic cycle of the parasite separate at different steps in the gradient.
Centrifuge

Centrifuge < 10,000 rpm
Supercentrifuge 10,000 – 20,000 rpm
Ultracentrifuge > 20,000 rpm

Ultracentrifuge

Preparative UC: separating the molecules according to their size or molar mass

Analytical UC: determination of the size or molar mass of the molecule
Electrophoresis is a technique in which charged molecules are separated according to physical properties such as charge or mass as they are forced through a matrix by an electrical current.
Movement of charged molecule in electrostatic field

What are the forces?
Coulomb-force:

$$F_c = QE = ZeE$$

$E=$electrostatic field
$e=$elementary charge
$Z=$the number of charges

Frictional force:

$$F_f = fv$$

$v =$velocity
$f =$shape factor

How long is the particle accelerating?

It can accelerate while:  

$$F_c = F_f$$ 

$$ZeE = fv$$
The electroforetic mobility

\[ u_{el} = \frac{v}{E} = \frac{Ze}{f} \]

means the velocity for unit electric field.

Supposing a sphere shape of the molecule:

\[ ZeE = 6\pi\eta rv \]

(Stokes-law)

\[ u_{el} = \frac{Ze}{6\pi\eta r} \]

Radius of the molecule can be calculated
Applications

The base of the separating techniques is that the molecules with different characteristics behave differently.

Types

• free flow electrophoresis
• gel electrophoresis
• capillary electrophoresis

For the electrophoresis we need: high voltage, electrodes, buffer and medium, which contains the buffer
This medium can be

• filter paper
• cellulose-acetate band
• different types of gel
• capillary
Free flow (boundary) electrophoresis

The electrophoretic tank: contains the solution with macromolecules;

Buffer is placed above this solution;

Voltage is put to the electrodes → electrophoresis
The boundary of the positively charged molecules in the left capillary is increasing

- the position of the moving boundary can be determined by applying optical measurements (absorption)

- height-concentration curve can be plotted $\rightarrow$ velocity can be calculated
The determination of the electrophoretic mobility

\[ u_{el} = \frac{v}{E} = \frac{Z e}{f} = \frac{Z e}{6 \pi \eta r} \]

*Can be measured*

**Calculation of the electric field from Ohm’s law.**

The resistance of a liquid column, where \( \Delta x \) is the height, \( A \) is the cross section, \( \rho = 1/\sigma \) is the resistivity or specific resistance, \( \sigma \) is the specific conductance

\[ \Delta R = \rho \frac{\Delta x}{A} = \frac{\Delta x}{\sigma A} \]

thus:

\[ E = \frac{\Delta U}{\Delta x \Delta x} = \frac{I \Delta R}{\Delta x} = \frac{I \Delta x}{\Delta x \sigma A} = \frac{I}{\sigma A} \]

*Can be calculated*
Gel electrophoresis

The separation components are in a gel: polyacrylamide, agarose...

- proteins
- giant proteins, DNA, RNA

- the running parameters are adjusted to the samples;
- the velocity depends on the size of the proteins.
The molecules must move in the same direction

\[ \text{SDS (sodium-dodecylsulfate)} \]

- anionic detergent: anionic polar end and a long apolar tail
- denaturizing agent
- it can bind the apolar part of the protein, make an electric interaction with the positive regions, the negative regions stay free
- all the proteins become negative \(\rightarrow\) separation of the molecules according to their size at a given pH (the small one is quicker, the big one is slower!)
- tracing of the run with coloured marker (e.g. bromophenole-blue) little molecular weight, so it runs ahead
PAGE
(polyacrylamide gel electrophoresis, 1959)

3D network \rightarrow \text{ pores}

**Acrylamide**

**Ammonium-persulfate:** initiates the polymerization

**TEMED:** free radicals, which catalyses the polymerisation

**Bis-acrylamide:** cross-link the neighbouring polymers and perform a rigid gel, its concentration determines the size of the pores
SDS-polyacrylamide gel

Staining (CoomassieBlue): After staining, different proteins will appear as distinct bands within the gel — photometry (Amido black, Fast green).
It is common to run molecular markers of known molecular weight in a separate lane in the gel and determine the weight of unknown proteins by comparing the distance traveled relative to the marker.

“Protocol silver staining” deposits metallic colloid silver onto the surface of a gel at the location of protein bands. Commercial silver stain kits are exceptionally robust and easy to use, detecting less than 0.5 nanograms of protein in typical gels, but requires very clear environment.
"Gradient gels" are specially prepared to have low percent-acrylamide at the top (beginning of sample path) and high percent-acrylamide at the bottom (end), enabling a broader range of protein sizes to be separated.

Along the lanes the density of the gel is increasing, so the faster proceeding proteins are slowed down by the greater resistance.

Production of gradient gels is similar to that of gradient elution buffers.
Isoelectric focusing

- separates the proteins based on their charges, or more precisely based on their pI points;
- proteins contain both positive and negative charge groups;
- If the pH changes, their net charge is changing as well;
  - at high pH-n: negative;
  - at low pH: positive.

**Isoelectric point**: the pH value where the protein has zero net charge;

- The electrophoresis is done in a pH gradient: the macromolecules move to their pI point and stop there loosing their net charge.
The application of the method

- pH gradient in a thin tube filled with gel;
- Electrophoresis in this medium;
- During the running the pH is changed around the protein and the net charge is decreasing.

- the proteins are running until they reach their corresponding pI point: the net charge becomes zero, the motion stops;
- equilibrium between electrophoresis and diffusion;
- the components with different pI values can be separated.
Two-dimensional electrophoresis

The principal: two separation methods are applied and they separate the sample proteins by two different sets of properties.

Perpendicular running of the methods:

1. Separation by isoelectric focusing (based on pI points);

2. *SDS-PAGE* gel electrophoresis (based on molecular weights).
The application of the method

Very good resolution!
An example for two-dimensional gel
First step: isoelectric focusing