

# Sedimentation and electrophoretic methods, Mass spectroscopy methods for pharmacy students

Dr. Tamás Bozó

assistant professor  
Department of Biophysics and Radiation Biology  
03.05.2022.



## Lecture topics

### Topics

- Sedimentation methods
  - Sedimentation
  - Sedimentation vs. Brownian motion
  - Centrifugation
    - Theory
    - Aspects
    - Categories
    - Devices
    - Methods
- Electrophoresis
  - Free flow electrophoresis
  - Gel electrophoresis
  - Isoelectric focusing
- Mass spectroscopy bases

### Related practice topics

- Diffusion
- Flow

### Textbook chapters

- VI/1.1. Sedimentation techniques
- VI/1.2. Electrophoresis and isoelectric focusing
- I/1.5; X/7. Mass spectrometry;



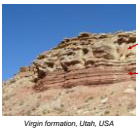
Department of Biophysics and Radiation Biology

2

## Sedimentation I.



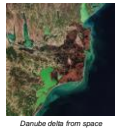
**Sedimentation** is the process of allowing particles in suspension to settle out of the suspension **under the effect of a force** (gravity, centrifugal acceleration, electromagnetism). The particles that settle out of the suspension become sediment.



Virgin formation, Utah, USA



Evolution of Danube delta (Romania) map



Danube delta from space



Department of Biophysics and Radiation Biology

3

## Sedimentation— II.

### Physical basis:

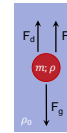
**Drag force** (fluid resistance) - a force acting opposite to the relative motion of any object moving with respect to a surrounding fluid.

$$F_d = f \cdot v$$

*This approximation is valid only for low velocities!*

$f$ : shape factor;  $f = \frac{1}{u}$   
 $v$ : speed  
 $u$ : mobility =  $\frac{v}{F}$

for a sphere:  
 $u = \frac{1}{6\eta r}$



**Bouyant force** (buoyancy) - an upward force exerted by a fluid that opposes the weight of a partially or fully immersed object.

$$F_b = \rho_0 \cdot V \cdot g$$

$$F_b = m \cdot g \cdot \frac{\rho_0}{\rho}$$

$$V = \frac{m}{\rho}$$

$\rho_0$ : density of the medium  
 $\rho$ : density of the particle  
 $V$ : particle volume  
 $m$ : particle mass  
 $g$ : gravity constant ( $9.8 \frac{m}{s^2}$ )

**Gravity force:**  
 $F_g = m \cdot g$



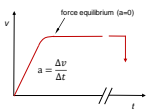
Department of Biophysics and Radiation Biology

4

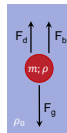
## Sedimentation – III.

### Physical basis:

**Newton's II. law:**  $\Sigma F = m \cdot a$



Particle velocity increases in time until the force equilibrium (or the bottom of the vessel) is reached.



Particle sediments in a lower density fluid

$$\Sigma F = F_b - F_d - F_g$$

At force equilibrium:  $\Sigma F = 0$

$$F_d = F_b - F_g$$

$$f \cdot v = m \cdot g - m \cdot g \cdot \frac{\rho_0}{\rho}$$

$$f \cdot v = m \cdot g \cdot \left(1 - \frac{\rho_0}{\rho}\right)$$

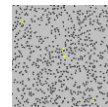


Department of Biophysics and Radiation Biology

5

## Sedimentation vs. Brownian motion

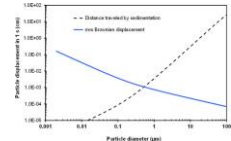
### Problem: Brownian motion



For small particles Brownian motion prohibit setting. Thus gravity-driven sedimentation will not work.

Particle (SC)	Diameter, microns	Brownian velocity, m/s	Sediment motion velocity, m/s
RBC	8	$1.5 \cdot 10^{-6}$	$5.5 \cdot 10^{-6}$
Latex ball	4	$5.5 \cdot 10^{-7}$	$1.7 \cdot 10^{-6}$
Latex ball	2	$1.6 \cdot 10^{-7}$	$5.3 \cdot 10^{-7}$
Latex ball	1	$4.4 \cdot 10^{-8}$	$1.1 \cdot 10^{-7}$
Milk fat particle	1	$5 \cdot 10^{-7}$	$2.7 \cdot 10^{-6}$
Latex ball	0.5	$1.2 \cdot 10^{-7}$	$2.7 \cdot 10^{-6}$

Chen et al. Romanian J. Biophys. 2010



Comparison of the root mean square Brownian displacement of a spherical particle (1000 kg/m³) and the distance traveled by sedimentation in air ( $p=1$  atm;  $T=293$  K)  
 Gensadarnes F Nanoeengineering, 2015

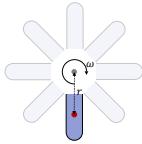


Department of Biophysics and Radiation Biology

6

## Centrifugation – theory I.

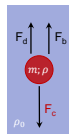
**Physical basis:** Sedimentation is forced by spinning



**Angular velocity:**

$$\omega = \frac{\Delta\phi}{\Delta t}$$

$\Delta\phi$  : angle taken by rotating object  
 $\Delta t$  : time



Particle sediments in a lower density fluid

**Drag force:**

$$F_d = f \cdot v$$

**Bouyant force:**

$$F_b = m \cdot a \cdot \frac{\rho_0}{\rho} = m \cdot r \cdot \omega^2 \cdot \frac{\rho_0}{\rho}$$

**Centrifugal force:**

$F_c = m \cdot a$  acceleration felt by the particle

$$F_c = m \cdot r \cdot \omega^2$$

$$a = r \cdot \omega^2$$
 distance from center (rotational radius)

At force equilibrium:  $F_d = F_c - F_b$

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

## Centrifugation – theory II.

At force equilibrium:  $F_d = F_c - F_b$

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



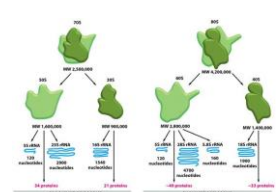
**Theodor Svedberg**  
1884-1971  
1926 Nobel Prize for Chemistry

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

**Sedimentation coefficient (S):**  
ratio of a particle's sedimentation velocity to the applied acceleration causing the sedimentation.

Unit: 1 Svedberg (Sv) =  $10^{-13}$  s

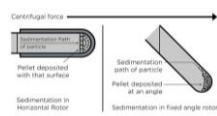
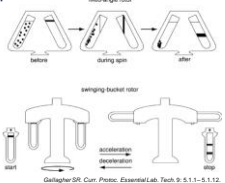
**An example: ribosome sedimentation**



## Centrifugation – aspects - I.

**Centrifugation:** An *analytical/preparatory* technique used for the separation of particles from a solution/suspension according to their **size, shape, density, density and viscosity of the medium, temperature and rotor speed**.

**Rotor types:**



[www.backman.com/resources/techniques/centrifugation/principles/rotor-types](http://www.backman.com/resources/techniques/centrifugation/principles/rotor-types)

Gallagher DR. Curr. Protoc. Essential Lab. Tech. 9.5.1.1-5.1.12.

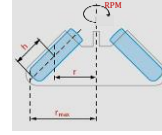
## Centrifugation – aspects - II.

**Relative centrifugal force (RCF):**

$$RCF = \frac{a}{g} = \frac{r \cdot \omega^2}{g}$$

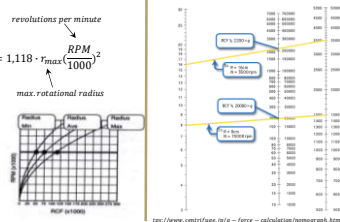
revolutions per minute

$$\text{Simplified formula: } RCF_{\text{max}} = 1.118 \cdot \frac{RPM^2}{10000} \cdot r_{\text{max}}$$



<https://handbook.solutions.appendix.com/>

**Nomograph for RCF determination.**



<http://www.centrifuge.org/g-force-calculation/nomograph.html>

## Centrifugation – categories – I.

	Analytical centrifugation	Preparative centrifugation
<b>Aim</b>	To study fundamental properties (mass, shape, interactions) of purified macromolecules or supramolecular assemblies.	To process biological samples for further analysis. To obtain/purify a particular component of a sample (pelletting).
<b>Examples</b>	<ul style="list-style-type: none"> <li>Determination of the purity (including the presence of aggregates) and oligomeric state of macromolecules;</li> <li>Determination of the average molecular mass of solutes in their native state;</li> <li>Study of changes in the molecular mass of supramolecular complexes;</li> <li>Detection of conformation and conformational changes.</li> </ul>	<ul style="list-style-type: none"> <li>Subcellular fractionation;</li> <li>Affinity purification of membrane vesicles;</li> <li>Separation of DNA components;</li> <li>Protein purification;</li> <li>Colloid separation;</li> <li>Virus purification;</li> </ul>
<b>Methods</b>	<ul style="list-style-type: none"> <li>Sedimentation velocity method;</li> <li>Sedimentation equilibrium studies</li> </ul>	<ul style="list-style-type: none"> <li>Differential centrifugation;</li> <li>Density gradient centrifugation (Rate-zonal or isopycnic)</li> </ul>

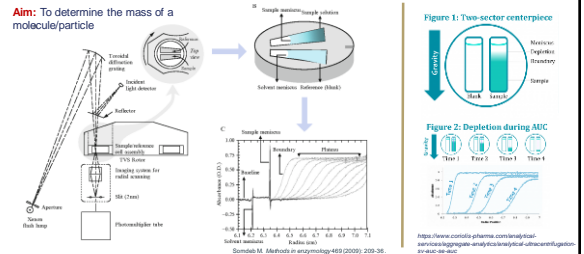
## Centrifugation – categories – II.

	Centrifugation	Ultracentrifugation
<b>Properties</b>	<ul style="list-style-type: none"> <li>Upper RCF limit approx. 65 000 g</li> <li>Refrigeration and vacuum are optional.</li> <li>Fractions are collected after the run.</li> </ul>	<ul style="list-style-type: none"> <li>High RCF-s (up to 1 000 000 g) - special rotors are needed</li> <li>At such high RPM-s friction would cause overheating and sample and/or device damage.</li> <li>Refrigeration and vacuum are mandatory.</li> <li>In-process detection</li> </ul>
<b>Examples</b>	<ul style="list-style-type: none"> <li>Separating cytosolic components from cell nuclei;</li> <li>Separating microparticles from a suspension.</li> </ul>	<ul style="list-style-type: none"> <li>Separating ribosomes, membrane vesicles, extracellular vesicles, proteins, DNA, viruses, etc.</li> </ul>
<b>Use</b>	Mostly preparative	Analytical OR Preparative

## Centrifugation – device examples

Centrifugation	Ultracentrifugation
 <p><b>Eppendorf 5427R</b></p> <ul style="list-style-type: none"> <li>RPM: max. 16.220</li> <li>RCF: max. 25.000 x g</li> <li>-10 °C to 40 °C</li> <li>30 kg</li> </ul>	 <p><b>Beckmann Coulter Optima XPN</b></p> <ul style="list-style-type: none"> <li>RPM: max. 100.000</li> <li>RCF: max. 802.400 x g</li> <li>0 °C to 40 °C</li> <li>485 kg</li> </ul>

## Sedimentation velocity method



## Sedimentation velocity method – II.

1. Ultracentrifuge to determine  $S$  from  $v$  and  $r\omega$

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

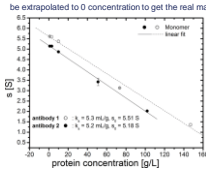
2. Express  $m$  as:  $m = \frac{fS}{(1 - \frac{\rho_0}{\rho})}$

3. To calculate  $m$  you need to know  $f$ ,  $\rho$  and  $\rho_0$

- $\rho_0$  can be calculated as  $\rho_0 = \frac{m_0}{V_0}$
- $\rho$  can be obtained by density gradient centrifugation (see later)
- $f$  can be derived from diffusion coefficient ( $D$ ):  $f = \frac{kT}{D} = \frac{RT}{ND}$

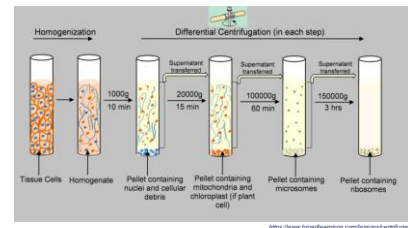
4. Express  $m$  as:  $m = \frac{RTS}{ND(1 - \frac{\rho_0}{\rho})}$

**Caveat:**  $S$  usually decrease with concentration  $\rightarrow S$  should be extrapolated to 0 concentration to get the real mass



## Differential centrifugation

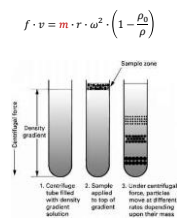
- Aim:** To separate components of a suspension



## Density gradient centrifugation – I.

### Rate Zonal Centrifugation

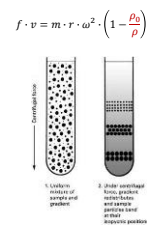
- Density gradient is formed in a centrifuge tube (e.g.: sugars, polymers, CsCl)
- Sample is layered on the top (max. 10%)
- Particles sediment at different rates according to their mass.
- Bands = particle fractions of identical mass (if density of the particles are the same)
- As  $\rho > \rho_0$  all particles sediment to the bottom if centrifuged too long
- Example: Separating of proteins and cellular organelles



## Density gradient centrifugation – II.

### Isopycnic Centrifugation

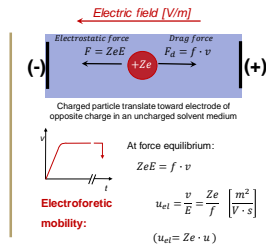
- Isopycnic = equal density
- Homogeneous mixture of the sample and gradient-forming material is placed in the tube
- Density gradient is formed during spinning
- Particles sediment or rise until they reach a layer of identical density.
- Bands = particle fractions of identical density
- After reaching equilibrium bands keep their position
- Example: Nucleic acids in CsCl



## Electroforetic methods

### Bases of electrophoresis

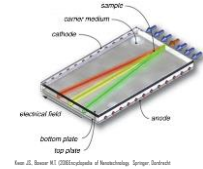
- Electrophoresis: translation due to electric field
- Biological molecules – usually charged at physiological conditions
- A charged molecule/particle will migrate in electric field
- Particles with asymmetric charge distribution become oriented in the field.
- Particles move with increasing velocity until  $F_{el}$  and  $F_d$  equilibrates – but not an equilibrium method
- Charges of the medium surrounds the travelling particle → largely affects mobility (retardation)
- Particles separate from each other due to their different mobility



## Electroforetic methods – II.

### Free flow electrophoresis

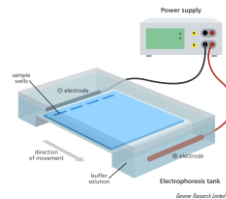
- Matrix-free electrophoretic separation technique
- Fluid flows between two plates to form a channel
- Perpendicular high voltage electric field is applied
- Laminar flow – fluid layers of different composition (pH; ionic strength etc.) can be applied
- Particles separate due to their charge density and/or isoelectric point
- Separation range: ions to cells
- Application: high-resolution separation of protein complexes, membrane proteins, protein and antibody isoforms, cells, subcellular compartments, etc.



## Electroforetic methods – III.

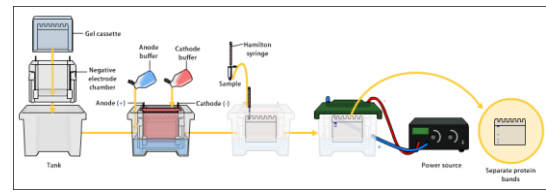
### Gel electrophoresis

- Matrix-based electrophoretic separation technique
- Semi-solid matrix (agar, polyacrylamide, starch...) – prevents heat convection (caused by electric field) and acts as a sieve → slows down the motion of particles.
- Small sample volumes
- High reproducibility
- High voltage electric field is applied
- Particles migrate and separate due to their size and charge.
- Separated fractions can be fixed, stained evaluated or extracted from further use
- Application: separation and analysis of macromolecules (DNA, RNA and proteins) and their fragments



## Electroforetic methods – IV.

### Gel electrophoresis - process



SDS-PAGE of recombinant E. coli RecA Protein

## Electroforetic methods – V.

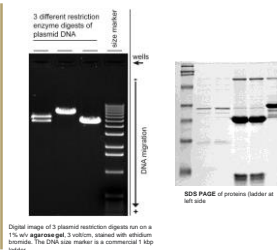
### Gel electrophoresis - gels

#### Agarose (0.5-3%)

- Natural polysaccharide polymers extracted from seaweed (macroalgae)
- Non-uniform pore-size
- Easy to handle
- Optimal for proteins (>200 kDa); DNA (from 50 bp)

#### Polyacrylamide

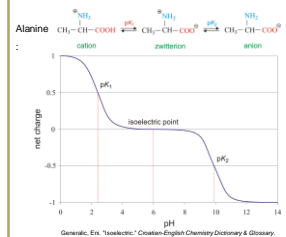
- Uniform pore size
- Polyacrylamide gel electrophoresis (PAGE) is used for separating proteins ranging in size from 5 to 2,000 kDa
- Proteins can be transferred onto a nitrocellulose or PVDF membrane → Western blot



## Electroforetic methods – V.

### Isoelectric focusing

- Certain macromolecules possess both acidic and basic residues → their net charge varies according to pH of their medium
- Isoelectric point: the pH at which the molecule has 0 net charge



## Electroforetic methods – VI.

### Isoelectric focusing

- Certain macromolecules possess both acidic and basic residues → their net charge varies according to pH of their medium
- Isoelectric point: the pH at which the molecule has 0 net charge
- Electrophoresis done in a medium with pH gradient → macromolecules migrate due to electric field until they reach their isoelectric point
- Here: equilibrium between diffusion and electrophoresis
- Molecules separate according to their isoelectric points
- High sensitivity
- Both for analytical and preparative purposes
- Used mostly for proteins

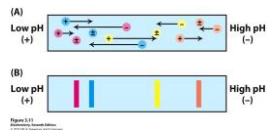
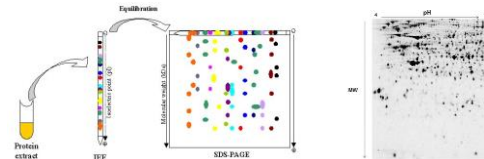


Figure 15.1  
Electrophoresis of proteins

## Electroforetic methods – VII.

### 2D- gel electrofocusing



<https://www.creative-proteomics.com/blog/index.php/two-dimensional-gel-electrophoresis-2-dg/>

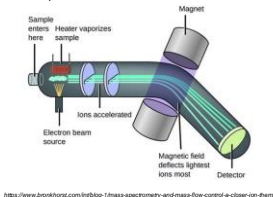
Mohand P. (2018) Difference Gel Electrophoresis Methods in Molecular Biology, vol 1054.

## Mass Spectrometry

### Bases in nutshell

- Used to determine the mass of ions in gas phase
- pM-aM sample quantities – even for trace analytics
- Main parts:
  - **Ion source:** transfers molecules to gas phase and ionizes them (eg.: Electron Spray Ionization, ESI; Matrix-Assisted Laser Desorption/Ionization, MALDI)
  - **Analyzer:** accelerates the ions, separates them based on m/z ratio using electric or magnetic field (eg.: quadrupole; Time-of-flight, TOF)
  - **Detector**
- Can be coupled to other analytical methods (LC-MS; GC-MS)

An example:

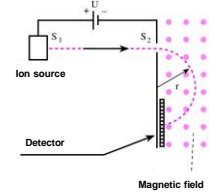


<https://www.biorxiv.org/content/10.1101/111111v1>

## Mass Spectrometry – II.

### Separation in magnetic field

- Electric field (with  $U$  accelerating voltage) accelerates the ions (of  $q$  charge) and provide them with a kinetic energy of:  $E_{kinetic} = Uq = \frac{1}{2} m \cdot v^2$
- Accelerated ions enter a magnetic field (induction lines are perpendicular to the direction of velocity). Lorentz force forces the ions to a circular path:  $F_{centrifugal} = \frac{m \cdot v^2}{r} = q \cdot v \cdot B$
- Radius can be calculated from position of the ion beam on the detector. Mass of the ion of interest:  $m = \frac{r^2 \cdot B^2 \cdot q}{2U}$



Thanks for your attention

Dr. Tamás Bozó