Sedimentation and electrophoretic methods, Mass spectroscopy methods

for pharmacy students

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SEMMELWEIS EGYETEM 1769

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

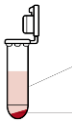
Textbook chapters

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

Sedimentation I.

Sedimentation is the process of allowing particles in suspension to settle out under the effect of a force.

Driving forces: gravity, centrifugal acceleration, electromagnetism.



Supernatant (liquid)

Pellet (precipitate, sediment)

https://handling-solutions.eppendorf.com/sample-handling/centrifugation/safe-use-of-centrifuges/basics-in-centrifugation/

Virgin formation, Utah, USA

Evolution of Danube delta (Romania) map

Danube delta from space

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.

$F_d = f \cdot v$

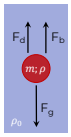
This approximation is valid only for low velocities!

f: *shape factor;* $f = \frac{1}{u}$

v: *speed*

u: *mobility* $= \frac{v}{F}$

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



Particle sediments in a lower density fluid

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

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

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

Gravity force:

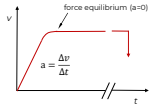
$F_g = m \cdot g$

ρ_0: *density of the medium*
ρ: *density of the particle*
V: *particle volume*
m: *particle mass*
g: *gravity constant* $(9.8\ \frac{m}{s^2})$

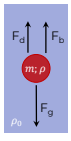
Sedimentation – III.

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

$\Sigma F = F_g - F_b - F_d$

At force equilibrium: $\Sigma F = 0$

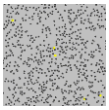
$F_d = F_g - F_b$

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

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

Sedimentation vs. Brownian motion

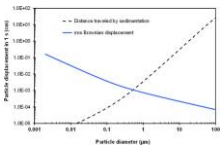
Problem: Brownian motion



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

Particle (SC)	Diameter, microns	Brownian velocity, m/s	Sediment motion velocity, m/s
RBC	8	$3.5*10^{-4}$	$3.5*10^{0}$
Latex ball	4	$5.5*10^{-5}$	$1.7*10^{-6}$
Latex ball	2	$1.6*10^{-5}$	$4.3*10^{-7}$
Latex ball	1	$4.4*10^{-6}$	$1.1*10^{-7}$
Milk fat particle	1	$5*10^{-5}$	$2.7*10^{-8}$
Latex ball	0.5	$1.2*10^{-6}$	$2.7*10^{-8}$

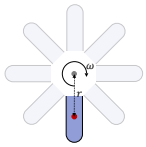
Chicea et al. Romanian J. Biophys. 2010

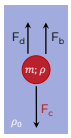


Comparison of the root mean square Brownian displacement of a spherical particle (1000 kg/m³) and the distance traveled by sedimentation in air (p=1 atm; T= 293 K) *Censdarmes F. Nanoengineering, 2015*

Centrifugation – theory I.

Physical basis: Sedimentation is forced by spinning





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:

$$F_c = m \cdot a \quad (a = r \cdot \omega^2) \quad \Rightarrow \quad F_c = m \cdot r \cdot \omega^2$$

(acceleration felt by the particle; distance from center (rotational radius))

Angular velocity:

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

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

Δt : time

Centrifugation – theory II.

At force equilibrium: $F_d = F_c - F_b$

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

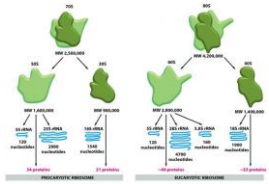
$$S \equiv \frac{v}{r \cdot \omega^2} = \frac{m}{f} \cdot \left(1 - \frac{\rho_0}{\rho}\right)$$

Theodor Svedberg
1884-1971
1926: Nobel Prize for Chemistry

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



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Further examples

Subcellular entity	Sedimentation coefficient (S)	Diameter (μm)
Nucleus	10^6 to 10^{12}	3–12[1]
Mitochondria	10^4 to 5×10^{4}[1]	0.5–4[1]
Lysosomes	4×10^3 to 2×10^{4}[1]	0.5–0.8[1]
Peroxisomes	4×10^{3}[1]	0.5–0.8[1]
Viruses	42 to >1000	0.02–0.4
Nucleic acids (free)	3.5 to 100	n/a
Ribosomes	80	0.025

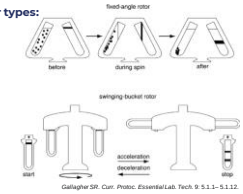
*Hinton and Mullock (1997)
†Schmidt (1973)
‡Luttmann et al. (2006)
§Griffith (1994)

Lawrence, Janice & Steward, Grieg. (2010). Purification of viruses by centrifugation.

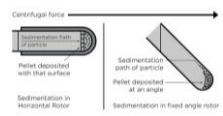
Centrifugation – aspects - I.

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

Rotor types:



Gallagher SR. Curr. Protoc. Essential Lab. Tech. 9: 5.1.1–5.1.12.



www.beckman.com/resources/technologies/centrifugation/principles/rotor-types

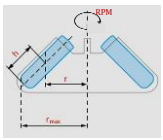
Centrifugation – aspects - II.

Relative centrifugal force (RCF):

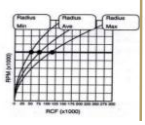
$$RCF = \frac{a}{g} = \frac{r \cdot \omega^2}{g}$$

Simplified formula: $RCF_{max} = 1{,}118 \cdot r_{max} \left(\frac{RPM}{1000}\right)^2$

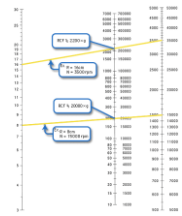
(revolutions per minute; max. rotational radius)



https://handling-solutions.eppendorf.com



Nomograph for RCF determination.



https://www.centrifuge.jp/g-force-calculation/nomograph.html

Centrifugation – categories – I.

	Analytical centrifugation	Preparative centrifugation
Aim	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 (pelleting).
Examples	• Determination of the purity (including the presence of aggregates) and oligomeric state of macromolecules; • Determination of the average molecular mass of solutes in their native state; • Study of changes in the molecular mass of supramolecular complexes; • Detection of conformation and conformational changes.	• Subcellular fractionation; • Affinity purification of membrane vesicles; • Separation of DNA components; • Protein purification; • Colloid separation; • Virus purification;
Methods	• Sedimentation velocity method; • Sedimentation equilibrium studies	• Differential centrifugation; • Density gradient centrifugation (Rate-zonal or isopycnic)

Centrifugation – categories – II.

	Centrifugation	Ultracentrifugation
Properties	• Upper RCF limit approx. 65 000 g • Refrigeration and vacuum are optional. • Fractions are collected after the run.	• High RCF-s (up to 1 000 000 g) - special rotors are needed • At such high RPM-s friction would cause overheating and sample and/or device damage. • Refrigeration and vacuum are mandatory. • In-process detection
Examples	• Separating cytosolic components from cell nuclei; • Separating microparticles from a suspension.	• Separating ribosomes, membrane vesicles, extracellular vesicles, proteins, DNA, viruses, etc.
Use	• Mostly preparative	• Analytical OR Preparative

Centrifugation – device examples

Centrifugation	Ultracentrifugation

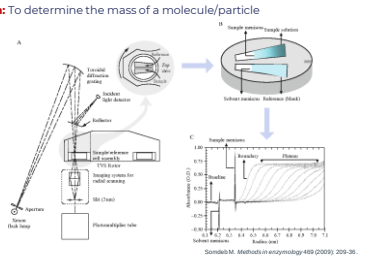
Eppendorf 5427R
- RPM: max. 16.220
- RCF: max. 25.000 x g
- -10 °C to 40 °C
- 30 kg

Eppendorf Minispin
- RPM: 800-13.400
- RFC: max. 12.100 x g
- Room temperature
- 4,3 kg

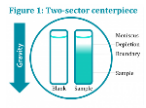
Beckmann Coulter Optima XPN
- RPM: max. 100.000
- RFC: max. 802.400 x g
- 0°C to 40 °C
- 485 kg

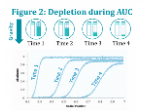
Sedimentation velocity method

Aim: To determine the mass of a molecule/particle



Somdeb M. Methods in enzymology 469 (2009): 209-36.





https://www.coriolis-pharma.com/analytical-services/aggregate-analytics/analytical-ultracentrifugation-sv-auc-se-auc

Sedimentation velocity method – II.

1. Ultracentrifuge to determine *S* from v and *r·ω* :

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

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

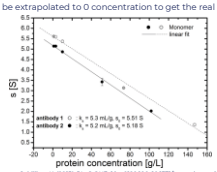
3. To calculate *m* you need to know *f*, *ρ* and ρ_0

- ρ_0: can be calculated as $\rho_0 = \frac{m_0}{V_0}$
- ρ: 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: $m = \frac{RTS}{ND(1 - \frac{\rho_0}{\rho})}$

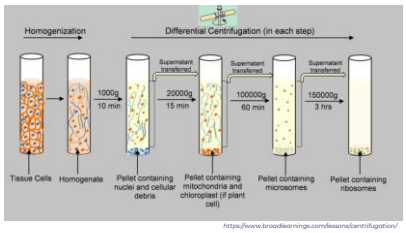
Caveat: S usually decrease with concentration → S should be extrapolated to 0 concentration to get the real mass



Schilling, K. (2015). PLoS ONE. 10. e0120820. 10.1371/journal.pone.0120820.

Differential centrifugation

Aim: To separate components of a suspension



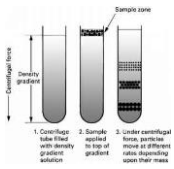
https://www.broadlearnings.com/lessons/centrifugation/

Density gradient centrifugation – I.

Rate Zonal Centrifugation

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

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

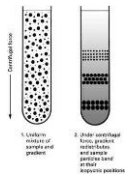


Density gradient centrifugation – II.

Isopycnic Centrifugation

- Isopycnic = equal density
- Homogeneous mixture of the sample and gradien-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
- Mass (size) and shape affects the sedimentation rate but not the final position
- After reaching equilibrium bands keep their position
- Example: nucleic acids in CsCl

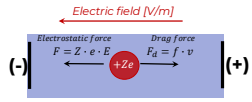
$$f \cdot v = m \cdot r \cdot \omega^2 \cdot \left(1 - \frac{\rho_0}{\rho}\right)$$



Electrophoretic 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* anf 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
- Application: analysis and preparation



Charged particle translate toward electrode of opposite charge in an uncharged solvent medium

At force equilibrium:

$$Z \cdot e \cdot E = f \cdot v$$

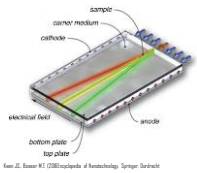
Electroforetic mobility: $u_{el} = \frac{v}{E} = \frac{Ze}{f}$ $\left[\frac{m^2}{V \cdot s}\right]$

$$(u_{el} = Ze \cdot u)$$

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.

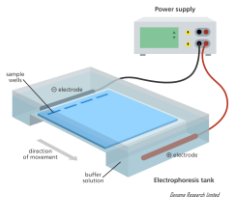


Kwon JE, Bowser MT. (2006) Encyclopedia of Nanotechnology. Springer. Dordrecht

Electroforetic methods – III.

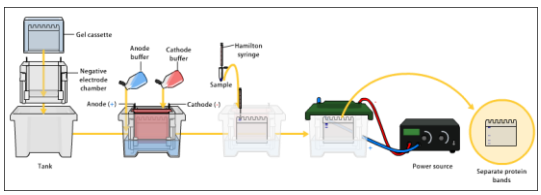
Gel electrophoresis

- 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



Kwon JE, Bowser MT. (2006) Encyclopedia of Nanotechnology. Springer. Dordrecht

SDS-PAGE of recombinant E. coli RecA Protein

Electroforetic methods – V.

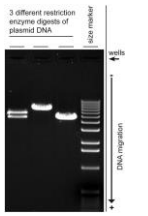
Gel electrophoresis - gels

Agarose (0,5-3%)

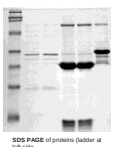
- Natural polysaccharide polymers extracted from seaweed (macroalgae)
- Non-uniform pore-size
- Easy to handle
- Optimal for proteins (>200 kDa); **DNA** (from 50 bp)

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



Digital image of 3 plasmid restriction digests run on a 1% w/v **agarose gel**, 5 volt/cm, stained with ethidium bromide. The DNA size marker is a commercial 1 kbp ladder..



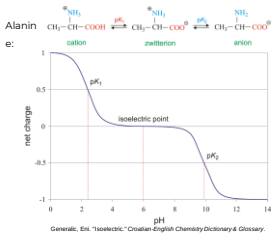
SDS PAGE of proteins (ladder at left side

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 wich the molecule has 0 net charge

Alanine:

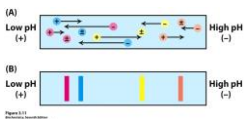


Generalic, Eni. "Isoelectric." Croatian-English Chemistry Dictionary & Glossary.

Electroforetic methods – VI.

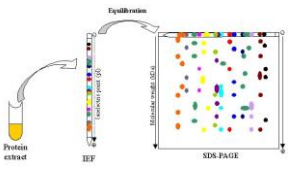
Isoelectric focusing

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

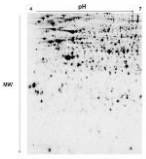


Electroforetic methods – VII.

2D- gel electroforesis



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



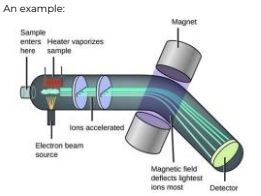
Meleady P. (2018) Difference Gel Electrophoresis. Methods in Molecular Biology, vol 1664.

Mass Spectrometry (MS)

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 mased 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)
- Further reading: textbook: X/7, I/1.5.

An example:



https://www.bronkhorst.com/ireblog-1/mass-spectrometry-and-mass-flow-control-a-closer-ion-them/

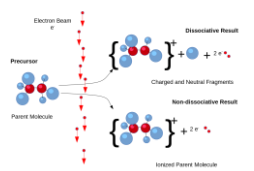
MS– Ionization methods I.

Electron ionization (EI)

- Sample is bombarded by electrons
- „Hard ionization": high energy elctrons → excessive fragmentation
- Most useful for organic compounds (MW<600)
- Formation of a molecular ion:

$$M + e^- \rightarrow M^+ + 2e^-$$

- Electron beam is produced by thermionic effect and acceleration in electric field.
- Advantages: simple, sensitive, fragments help molecule identification, library-searchable fingerprint spectra
- Disadvantages: only volatile and thermally stable molecules, excessive fragmentation, MW < 1000
- Frequently coupled with Gas Chromatography (GC-MS)

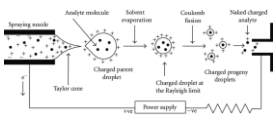


By Evan Mason - Own work, CC BY-SA 4.0, https://commons.wikimedia.org/w/index.php?curid=37926204

MS– Ionization methods II.

Electrospray ionization (ESI)

- Sample to be analyzed is dispersed in a liquid
- Aerosol is produced
- High voltage is applied
- „Soft ionization" → low propensity of fragmentation → especially useful to produce molecular ions from macromolecules.

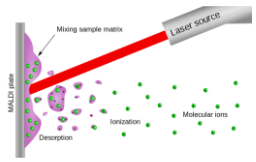


- Advantages: accurate, fast, broad mass range, low fragmentation
- Disadvantages: no structural information (in lack of fragments)
- Can be coupled to Liquid Chromatography (LC-MS)

MS– Ionization methods III.

Matrix Assisted Laser Desorption Ionization (MALDI)

- Sample is mixed with an energy-absorbing matrix
- Laser pulse triggers sample and matrix ablation and desorption
- Analyte molecules become ionized in the gas phase
- Advantages: accurate, fast, broad mass range, soft ionization, not just for volatile samples, sub-picomole sensitivity
- Disadvantages: expensive instrumentation
- Most widely used with time-of-flight (TOF) spectrometers



MS – Mass analyzers I.

Separation in magnetic field

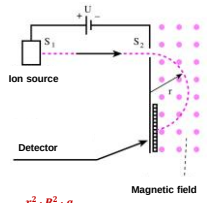
- Electric field (with U accelerating voltage) accelerates the ions (of q charge) and provide them with a kinetic energy of:

$$E_{kinetic} = U \cdot q = \frac{1}{2} m \cdot v^2$$

- Accelerated ions enter a magentic field (induction lines are perpendicular to the direction of velocity). Lorentz force forces the ions to a circular path:

$$F_{centipretal} = \frac{m \cdot v^2}{r} = q \cdot v \cdot B$$

- Radius can be calculated from position of the ion beam on the detector. Mass of the ion of interest:

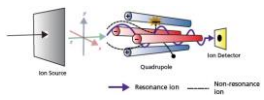


$$m = \frac{r^2 \cdot B^2 \cdot q}{2U}$$

MS – Mass analyzers II.

Quadropule mass analyzer

- 4 parallel, cylindrical rod
- Oscillating electric field is applied on the rods – opposing rods are connecter electrically → quadropole is formed
- Ions travel through the rods
- Only ions of a certain mass-to-charge ratio will reach the detector for a given ratio of voltages
- Other ions have unstable trajectories and will collide with the rods.
- By varying the voltage a wide m/z range can be scanned

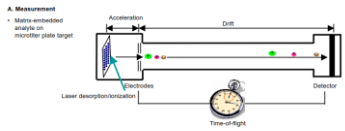


MS – Mass analyzers III.

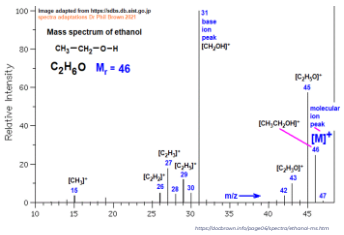
Time-of-flight (TOF) mass analyzer

- m/z ratio is measured by the time of arrival to the detector

$$E = qU = \frac{1}{2} m v^2$$



Mass Spectrum



- Histogram plot of intensity vs. m/z
- m/z: mass-to-charge ratio
- z (charge number is used instead of q (charge)
- Molecular ion peak + fragments
- Fragmentation depends on the type of spectrometer
- Isotope peaks may appear
- Spectral analysis → sample composition

Thanks for your attention

Dr. Tamás Bozó

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