Centrifugation Techniques

By- Dr. Ekta Khare

INTRODUCTION

- Biological centrifugation is a process that uses centrifugal force (Centrifugal force is a pseudo force in a circular motion which acts along the radius and is directed away from the centre of the circle) to separate and purify mixtures of biological particles in a liquid medium.
- It is a key technique for isolating and analysing cells, subcellular fractions, supramolecular complexes and isolated macromolecules such as proteins or nucleic acids.
- In 1864, Antonin Prandtl invented the first centrifuge-type machine, which was used in the dairy industry to separate milk and cream on a large scale.
- Following Prandtl, Friedrich Miescher, a Swiss physician and biologist, was the first to apply centrifugation in the lab(1869).
- The development of the **first analytical ultracentrifuge by Svedberg in the late 1920s** and the technical refinement of the preparative centrifugation technique by **Claude and colleagues in the 1940s** positioned centrifugation technology at the centre of biological and biomedical research for many decades.
- Today, centrifugation techniques represent a critical tool for modern biochemistry and are employed in almost all invasive subcellular studies.
- While analytical centrifugation is mainly concerned with the study of purified macromolecules or isolated supramolecular assemblies, preparative centrifugation methodology is devoted to the actual separation of tissues, cells, subcellular structures, membrane vesicles and other particles of biochemical interest.

BASIC PRINCIPLES OF SEDIMENTATION

- When designing a centrifugation protocol, it is important to keep in mind that:
  - the more dense a biological structure is, the faster it sediments in a centrifugal field;
  - the more massive a biological particle is, the faster it moves in a centrifugal field;
  - the denser the biological buffer system is, the slower the particle will move in a centrifugal field;
  - the greater the frictional coefficient is, the slower a particle will move (The frictional coefficient depends on the size and shape of the biological particle. The frictional force of a particle moving through a viscous fluid is the product of its velocity and its frictional coefficient, and acts in the opposite direction to sedimentation.);
  - the greater the centrifugal force is, the faster the particle sediments;
  - the sedimentation rate of a given particle will be zero when the density of the particle and the surrounding medium are equal.

… BASIC PRINCIPLES OF SEDIMENTATION

- Essentially, the rate of sedimentation is dependent upon the applied centrifugal field (cm s^{-2}), G, that is determined by the radial distance, r, of the particle from the axis of rotation (in cm) and the square of the angular velocity, ω, of the rotor (in radians per second):

$$G = \omega^2 r$$

- The average angular velocity of a rigid body that rotates about a fixed axis is defined as the ratio of the angular displacement in a given time interval.
- One radian, usually abbreviated as 1 rad, represents the angle subtended at the centre of a circle by an arc with a length equal to the radius of the circle.
- Since 360^o equals 2π radians, one revolution of the rotor can be expressed as 2 π rad.
- Accordingly, the angular velocity in rads per second of the rotor can be expressed in terms of rotor speed s as:

$$\omega = \frac{2\pi s}{60}$$

- and therefore the centrifugal field can be expressed as: $$G = \frac{4\pi^2(\text{rev min}^{-1})^2 r}{3600} = \frac{4\pi^2 s^2 r}{3600}$$

Example 1 CALCULATION OF CENTRIFUGAL FIELD

Question What is the applied centrifugal field at a point equivalent to 5 cm from the centre of rotation and an angular velocity of 3000 rad s^{-1}?

Answer The centrifugal field, G, at a point 5 cm from the centre of rotation may be calculated using the equation

$G = \omega^2 r = (3000)^2 \times 5\ \mathrm{cm\,s^{-2}} = 4.5 \times 10^7\ \mathrm{cm\,s^{-2}}$

Example 2 CALCULATION OF ANGULAR VELOCITY

Question For the pelleting of the microsomal fraction from a liver homogenate, an ultracentrifuge is operated at a speed of 40 000 r.p.m. Calculate the angular velocity, $\boldsymbol{\omega}$, in radians per second.

Answer The angular velocity, $\boldsymbol{\omega}$, may be calculated using the equation:

$$\omega = \frac{2\pi\ \mathrm{rev\ min^{-1}}}{60}$$

$\omega = 2 \times 3.1416 \times 40{,}000/60\ \mathrm{rad\,s^{-1}} = 4188.8\ \mathrm{rad\,s^{-1}}$

… BASIC PRINCIPLES OF SEDIMENTATION

- The centrifugal field is generally expressed in multiples of the gravitational field, g (981 cm s^{-2}).
- The relative centrifugal field (g), RCF, which is the ratio of the centrifugal acceleration at a specified radius and the speed to the standard acceleration of gravity, can be calculated from the following equation:

$$\mathrm{RCF} = \frac{4\pi^2(\mathrm{rev\ min}^{-1})^2 r}{3600 \times 981} = \frac{G}{g}$$

$$\mathrm{RCF} = 1.12 \times 10^{-5}\ \mathrm{r.p.m.}^2 r.$$

Example 3 CALCULATION OF RELATIVE CENTRIFUGAL FIELD

Question A fixed-angle rotor exhibits a minimum radius, $r_{\min}$, at the top of the centrifuge tube of 3.5 cm, and a maximum radius, $r_{\max}$, at the bottom of the tube of 7.0 cm. If the rotor is operated at a speed of 20 000 r.p.m., what is the relative centrifugal field, RCF, at the top and bottom of the centrifuge tube?

Answer The relative centrifugal field may be calculated using the equation:

$$\mathrm{RCF} = 1{,}12 \times 10^{-5}\ \mathrm{r.p.m.}^2 r$$

Top of centrifuge tube:

$$\mathrm{RCF} = 1{,}12 \times 10^{-5} \times (20\,000)^2 \times 3.5 = 15\,680$$

Bottom of centrifuge tube:

$$\mathrm{RCF} = 1{,}12 \times 10^{-5} \times (20\,000)^2 \times 7.0 = 31\,360$$

This calculation illustrates that with fixed-angle rotors the centrifugal field at the top and bottom of the centrifuge tube might differ considerably, in this case exactly two-fold.

Stokes' Law

- In a suspension of biological particles, the rate of sedimentation is dependent not only upon the applied centrifugal field, but also on the nature of the particle, i.e. its density and radius, and also the viscosity of the surrounding medium.
- Stokes' Law describes these relationships for the sedimentation of a rigid spherical particle:

$$\nu = \frac{2}{9}\frac{r^2(\rho_{\mathrm{p}} - \rho_{\mathrm{m}})}{\eta} \times g$$

- where ν is the sedimentation rate of the sphere, 2/9 is the shape factor constant for a sphere, r is the radius of particle, ρ_p is the density of particle, ρ_m is the density of medium, g is the gravitational acceleration and η is the viscosity of the medium.

… BASIC PRINCIPLES OF SEDIMENTATION

- The sedimentation rate or velocity of a biological particle can also be expressed as its sedimentation coefficient (s), whereby:

$$s = \frac{\nu}{\omega^2 r}$$

- Since the sedimentation rate per unit centrifugal field can be determined at different temperatures and with various media, experimental values of the sedimentation coeffificient are corrected to a sedimentation constant theoretically obtainable in water at 20° C, yielding the $S_{20,W}$ value.
- The sedimentation coefficients of biological macromolecules are relatively small, and are usually expressed, as Svedberg units, S.
- One Svedberg unit equals 10^{-13} s.

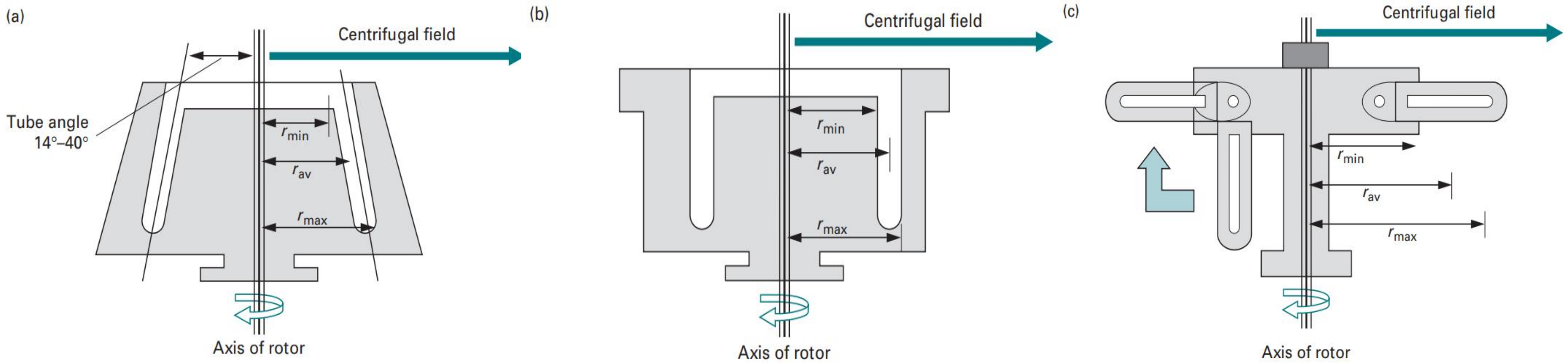
Types of centrifuges

- The most obvious differences between centrifuges are:
  - the maximum speed at which biological specimens are subjected to increased sedimentation;
  - the presence or absence of a vacuum;
  - the potential for refrigeration or general manipulation of the temperature during a centrifugation run; and
  - the maximum volume of samples and capacity for individual centrifugation tubes.
- Many different types of centrifuges are commercially available including:
  - large-capacity low-speed preparative centrifuges;
  - refrigerated high-speed preparative centrifuges;
  - analytical ultracentrifuges;
  - preparative ultracentrifuges;
  - large-scale clinical centrifuges; and
  - small-scale laboratory microfuges.

Types of rotors

- Depending on the use in a simple low-speed centrifuge, a high-speed centrifuge or an ultracentrifuge, different centrifugal forces are encountered by a spinning rotor.
- Accordingly different types of rotors are made from different materials.
- Low-speed rotors are usually made of steel or brass, while high-speed rotors consist of aluminium, titanium or fibre-reinforced composites.
- The exterior of specific rotors might be finished with protective paints.
- For example, rotors for ultracentrifugation made out of titanium alloy are covered with a polyurethane layer.
- Aluminium rotors are protected from corrosion by an electrochemically formed tough layer of aluminium oxide.
- In order to avoid damaging these protective layers, care should be taken during rotor handling.

Design of the three main types of rotors used in routine biochemical centrifugation techniques. Shown is a cross-sectional diagram of a fixed-angle rotor (a), a vertical tube rotor (b), and a swinging-bucket rotor (c). A fourth type of rotor is represented by the class of near-vertical rotors (not shown).



…Rotors

- **Fixed-angle rotors** are an ideal tool for pelleting during the differential separation of biological particles where sedimentation rates differ significantly, for example when separating nuclei, mitochondria and microsomes.
- In addition, isopycnic banding may also be routinely performed with fixed-angle rotors.
- For isopycnic separation, centrifugation is continued until the biological particles of interest have reached their isopycnic position in a gradient.
- This means that the particle has reached a position where the sedimentation rate is zero because the density of the biological particle and the surrounding medium are equal.
- Centrifugation tubes are held at a fixed angle of between 14 ^{o} and 40 ^{o} to the vertical in this class of rotors.
- **Vertical rotors** may be divided into true vertical rotors and near-vertical rotors.
- Sealed centrifuge tubes are held parallel to the axis of rotation in vertical rotors and are restrained in the rotor cavities by screws, special washers and plugs.
- Since samples are not separated down the length of the centrifuge tube, but across the diameter of the tube, isopycnic separation time is significantly shorter as compared to swinging bucket- rotors.

…Rotors

- In contrast to fixed-angle rotors, **near-vertical rotors** exhibit a reduced tube angle of 7^o to 10^o and also employ quick-seal tubes.
- The reduced angle results in much shorter run times as compared to fixed-angle rotors.
- Near-vertical rotors are useful for gradient centrifugation of biological elements that do not properly participate in conventional gradients.
- Hinge pins or a crossbar is used to attach rotor buckets in **swinging-bucket rotors**.
- They are loaded in a vertical position and during the initial acceleration phase, rotor buckets swing out horizontally and then position themselves at the rotor body for support.
- Since a greater variety of gradients exhibiting different steepness can be used with swinging-bucket rotors, they are the method of choice when maximum resolution of banding zones is required.

Operation of the three main types of rotors used in routine biochemical centrifugation techniques. Shown is a cross-sectional diagram of a centrifuge tube positioned in a fixed-angle rotor (a), a vertical tube rotor (b), and a swinging-bucket rotor (c).