

BMS COLLEGE OF ENGINEERING, BANGALORE-19
DEPARTMENT OF BIOTECHNOLOGY
Faculty : Dr. V. Saisha

BIOPHYSICAL TECHNIQUES

Topics:

Rayleigh Scattering, ultra centrifugation, viscometry, electron microscopy (SEM,TEM,AFM),luminescence (fluorescence and phosphorescence),calorimetry and DSC, Mass spectroscopy, LCMS,MALDI-TOF, voltage clamp and patch clamp (measurement of membrane potentials)

RAYLEIGH SCATTERING

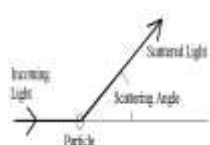
Scattering fundamentals

Scattering can be broadly defined as the redirection of radiation out of the original direction of propagation, usually due to interactions with molecules and particles. The radiation is treated according to the classical electromagnetic theory.this is appropriate for scattering studies , where trabsitions between quantized energy states are not involved.

Matter is composed of discrete electrical charges (atoms and molecules – dipoles) Light is an oscillating EM field – excites charges, which radiate EM waves.These radiated EM waves are scattered waves, excited by a source external to the scatterer.The superposition of incident and scattered EM waves is what is observed.

Scattering geometry

Light when incident on a particle gets scattered in all directions. The angle at which the light ray is shifted/ deflected from the path of the incident ray is called as the scattering angle.



When does scattering matter?

Scattering can be ignored whenever gains in intensity due to scattering along a line of sight are negligible compared to:

Losses due to extinction

Gains due to thermal emission

Usually satisfied in the thermal IR band and for microwave radiation when no precipitation (rain, snow etc.) is present Also can be ignored when considering direct radiation from a point source, such as the sun. In the UV, visible and near-IR bands, scattering is the dominant source of radiation along any line of sight, other than that looking directly at the sun

Types of scattering

Elastic scattering – the wavelength (frequency) of the scattered light is the same as the incident light (Rayleigh and Mie scattering)

Inelastic scattering – the emitted radiation has a wavelength different from that of the incident radiation (Raman scattering, fluorescence)

BMS COLLEGE OF ENGINEERING, BANGALORE-19
DEPARTMENT OF BIOTECHNOLOGY
Faculty : Dr. V. Saisha

Quasi-elastic scattering – the wavelength (frequency) of the scattered light shifts (e.g., in moving matter due to Doppler effects)

Parameters governing scattering

1) The wavelength (λ) of the incident radiation

(2) The size of the scattering particle, usually expressed as the non-dimensional size parameter,

$$x = \frac{2\pi r}{\lambda}, \text{ , } r \text{ is the radius of a spherical particle, } \lambda \text{ is wavelength}$$

(3) The particle optical properties relative to the surrounding medium: the complex refractive index

Scattering regimes:

$x \ll 1$: Rayleigh scattering

$x \sim 1$: Mie scattering

$x \gg 1$: Geometric scattering

Applications of the principle of Rayleigh scattering.

Consider that a molecule is at position $x=0$, $y=0$ and $z=0$ (origin) and monochromatic light is directed along x axis -and polarized with its electric vector in z axis.

The eqn. for electric vector at point x and time t is

$$E(x,t) = E_0 \cos 2\pi (vt - x/\lambda), \text{ At } x=0$$

$$E(0,t) = E_0 \cos 2\pi vt$$

According to classical electromagnetic theory, this oscillation of the external field should produce corresponding oscillation of the electrons within the molecule.

Such oscillating charges will cause the molecule to have an oscillating dipole, which will in turn act as a minute antenna, dispersing some of the energy in directions other than the direction of the incident radiation.

The scattered radiation arises from the oscillating dipole moment, a vector determined by the molecular polarizability, α of the molecule.

$$\text{Given as } \mu = \alpha E = \alpha E_0 \cos 2\pi vt$$

The instantaneous amplitude of the electric field produced by an oscillating dipole, observed at a distance y from the dipole and at an angle θ with respect to the direction of polarization (the z -axis), is given by electromagnetic theory as

$$E_r = [\alpha E_0 4\pi^2 \sin\theta / r\lambda^2] \cos 2\pi (vt - r/\lambda)$$

This eqn. gives information on amplitude of scattered light. The intensity of the radiation (the energy flow per square centimeter) depends on the square of the amplitude. The intensity of scattering increases rapidly with decreasing wavelength. The intensity depends on the angle θ ; there is no radiation along the direction ($\theta = 0$) in which the dipole vibrates

Intensity of scattered light can be given as

BMS COLLEGE OF ENGINEERING, BANGALORE-19
DEPARTMENT OF BIOTECHNOLOGY
Faculty : Dr. V. Saisha

$$\frac{i}{I_0} = \frac{8\pi^4 \alpha^2}{r^2 \lambda^4} (1 + \cos^2 \theta)$$

Eqn. giving description of the scattering of light by a single small, isotropic particle
 Eqn not applicable for study solutions containing many Macromolecules i.e scattering is by a collection of particles.

Scattering from a Number of Small Particles: Rayleigh Scattering : assumptions :

solution contains N identical molecules per cm³ .,The molecules are smaller than the wavelength of the radiation.,Solution is so dilute that inter particle interference effects are negligible.

Under these conditions, the intensity of the light scattered by these particles (in excess of that scattered by solvent) will just be the sum of the intensities for all the particles.

$$\frac{i}{I_0} = \frac{8\pi^4 \alpha^2}{r^2 \lambda^4} (1 + \cos^2 \theta)$$

-----eqn.1

where n is the refractive index of the solution and n₀ is the refractive index of the solvent
 it is necessary to express the polarizability, α, in terms of some more easily measurable quantity.
 the refractive index provides such a quantity

$$n^2 - n_0^2 = 4\pi N \alpha$$

The relationship is :

where n is the refractive index of the solution and n₀ is the refractive index of the solvent

Rearranging and multiplying and dividing by the weight concentration C (gm/ml)

$$\alpha = (n - n_0)(n + n_0)C / 4\pi CN$$

At low concentrations n + n₀ == 2 n₀, and

(n - n₀)/C == dn/dC, where dn/dC is an

easily measured change in n with a change in C, called the specific refractive index increment. So,

$$\alpha = n_0 / 2 \pi (dn/dC) C / N$$

Substituting in eqn.1 , we get

$$\frac{i_{\theta}}{I_0} = \frac{2\pi^2 n_0^2}{r^2 \lambda^4} \left(\frac{dn}{dC} \right)^2 \frac{C^2}{N} (1 + \cos^2 \theta)$$

note that N = CK / M, where K is Avogadro's number and M is the molecular weight of the macromolecule

Substituting in eqn. , we get

$$\frac{i_{\theta}}{I_0} = \frac{2\pi^2 n_0^2 (dn/dC)^2}{r^2 \lambda^4 N} CM (1 + \cos^2 \theta)$$

BMS COLLEGE OF ENGINEERING, BANGALORE-19
DEPARTMENT OF BIOTECHNOLOGY
Faculty : Dr. V. Saisha

The above eqn. tells us that the excess scattering produced by a solution containing a weight concentration C of particles of molecular weight M depends on the product CM .

It also depends on the angle with respect to the incident beam, θ , but it is symmetrical regarding forward and backward scattering, if the scattering particles are small compared to the wavelength of the light.

For such scattering, which is called Rayleigh scattering, we can define a quantity, the Rayleigh ratio, which corrects for the $1 + \cos^2 \theta$ term in Eq. and is therefore independent of angle.

$$R_{\theta} = \frac{i_{\theta}}{I_0} \frac{r^2}{(1 + \cos^2 \theta)}$$

Substituting and rearranging ,

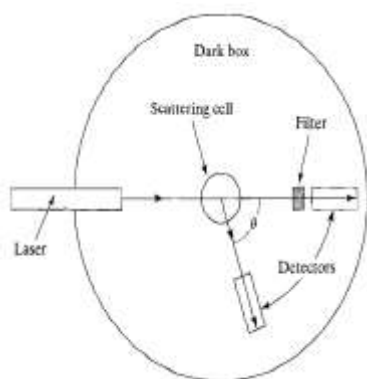
$$R_{\theta} = \frac{2\pi^2 n_0^2 (dn/dC)^2}{N\lambda^4} CM = KCM \quad K = \frac{2\pi^2 n_0^2 (dn/dC)^2}{N\lambda^4}$$

These equations show that light-scattering measurements can be used for molecular-weight determination. With real solutions, the equations must be modified to take into account the non ideality of the solution. Ideality was implied when we said that the scattering of N particles was N times the scattering from a single particle; this will be true only if the particles are entirely independent of one another

. If there are correlations between their positions (if even, for example, the center of one particle cannot penetrate into the space occupied by another), the scattering will be modified by this solution nonideality The measurement of R_0 is usually carried out in a photometer

The intensity of light scattered at a given angle is compared with the intensity of the incident light. In addition to R_0 values, calculation of the molecular weight requires knowledge of dn/dC . Since the difference in refractive index between a dilute solution and pure solvent is very small, a differential refractometer, which directly measures the small difference $n - n_0$, is commonly used.

Schematic design of an instrument for light-scattering measurements.



BMS COLLEGE OF ENGINEERING, BANGALORE-19
DEPARTMENT OF BIOTECHNOLOGY
Faculty : Dr. V. Saisha

A continuous wave laser is used as the light source, and the output from the scattering detector (which can rotate about the cell) is compared with that from the direct beam detector. A filter is inserted to reduce the intensity for the latter.

Scattering from Particles That Are Not Small Compared to Wavelength of Radiation

So far, we have assumed that the greatest dimension of the scattering particle is small compared to the wavelength of the light. There are two kinds of situations in which this will not be true.

- 1) very large particles like viruses and immense proteins or nucleic acids are not insignificant in dimensions compared to visible light.
- 2) Even small proteins can have dimensions much larger than the wavelengths of such radiation (in case of X-rays or neutron)

In such cases ;. In addition to the measurement of mass, it becomes possible to estimate dimensions and even obtain some information about shape or internal structure of the molecules.

Since the scattering centers within a given large molecule are (more or less) fixed with respect to one another, we can no longer consider them to be independent scatterers. We must therefore take into account interference between radiation from these scattering centers. For convenience, we express the general result by defining a function $P(\theta)$, which describes how the scattering at angle θ corresponds to that which the same molecule would have were its dimensions shrunk to be much smaller than λ . Given as :

$$P(\theta) = \frac{1}{n^2} \sum_{i=1}^n \sum_{j=1}^n 1 = \frac{n^2}{n^2} = 1$$

Thus, at very low angles, at very long wavelengths, or for very small particles, Rayleigh scattering is approached.

Its use is limited at one extreme by the low scattering power of small molecules and at the other by the difficulties encountered in the interpretation for very large macromolecules.

BMS COLLEGE OF ENGINEERING, BANGALORE-19
DEPARTMENT OF BIOTECHNOLOGY
Faculty : Dr. V. Saisha

ULTRACENTRIFUGATION

Centrifugation is one of the most important and widely applied research techniques in Biochemistry, Cellular and Molecular biology, and in Medicine. The centrifuge works using the sedimentation principle, where the centripetal acceleration causes more dense substances to separate out along the radial direction (the bottom of the tube).

Sedimentation

When particles are forced through a solution, they experience resistance to movement which depends on properties of the particle such as mass, shape and density and properties of the solvent such as its temperature, viscosity, density and composition. A commonly-used experimental format where this occurs is sedimentation in a centrifugal field which is also called centrifugation. This can be used as a preparative strategy to separate complex mixtures present in biological samples. Alternatively, it can also be used analytically to determine the mass, shape or density of particles.

In 1926 Theodor Svedberg (1884-1971) received a Nobel Prize for his work in colloid chemistry and invention of the ultracentrifuge..

Physical Basis of Centrifugation

Consider a particle of mass, m_0 , suspended in a solvent of density, ρ . This particle would experience an upthrust equivalent to the weight of displaced liquid in accordance with Archimedes's principle. The buoyant mass, m , of the particle is therefore m_0 minus a correction factor for this up thrust

$m = m_0 - m_0 \cdot v \cdot \rho$ -----1 where v is the partial specific volume of the particle.

This is equal to the volume displaced by the particle and is approximately equal to the reciprocal of the density of the solute although the degree of particle hydration can affect it. For example, charged particles (which attract solvent molecules, compacting them in its vicinity) give smaller values of v than might be anticipated from particle density alone

If this particle is exposed to a centrifugal field, it experiences a centrifugal force, F ;

$F = m \cdot \omega^2 \cdot r$ -----2 where ω is the angular velocity of rotation (in units of radians/sec. with one revolution = 2π radians) and r is the distance of the particle from the centre of rotation (cm).

Combining Equations 1&2 gives us;

BMS COLLEGE OF ENGINEERING, BANGALORE-19
DEPARTMENT OF BIOTECHNOLOGY
Faculty : Dr. V. Saisha

$$F = m\omega^2 r (1 - v \cdot \rho) \quad \text{-----3}$$

This equation means that, as the mass, angular velocity or distance from the centre of rotation increases, so does the centrifugal force experienced by a particle. As the particle passes through the solvent it experiences resistance due to a frictional force, F^* , operating in the opposite direction

$F^* = f \cdot v$ -----4 where f is a frictional coefficient dependent on the shape and mass of the particle and the viscosity of the solvent and v is the velocity of the particle. At the beginning of centrifugation, the particle accelerates through the solvent but eventually the frictional force decreases this acceleration such that a constant velocity called the sedimentation velocity is achieved. At this point; $F = F^*$

i.e. $m\omega^2 r (1 - v \cdot \rho) = f \cdot v$ The sedimentation velocity is given by;

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

Sedimentation velocity is dependent on the shape of the particle but, for a perfect sphere, this quantity is related to physical properties of the particle and solvent by;

$$v = \frac{a^2 [\rho_p - \rho_m] \omega^2 r}{18\eta} \quad \text{-----6}$$

where a is the particle diameter, ρ_p and ρ_m are the densities of the particle and solvent, respectively and η is the viscosity of the solvent. The inverse relationship with viscosity means that sedimentation velocity decreases markedly with increase in solvent viscosity and (since viscosity is dependent on temperature) with temperature. For this reason, sedimentation velocities are usually corrected for different solvent composition and temperature to standard conditions of pure water at 20 °C.

The relationship described by Equation (6) underlies all centrifugation experiments in biochemistry and is the reason why different particles (i.e. with differing partial specific volumes and mass) can achieve individual sedimentation velocities in a single solvent at a single angular velocity. It provides the basis for differential centrifugation in which particles may be conveniently separated from each other. It is difficult to determine v , ρ and f accurately so a useful measure of differential behavior in a centrifugal field is provided by the sedimentation coefficient, s

$$s = \frac{v\omega^2 r}{f} = \frac{m(1 - v \cdot \rho)}{Nf}, \quad \text{as } m = \frac{M}{N}, \quad v = \text{vol. occupied by 1gm of solute.}$$

or

$$\frac{M(1 - \bar{v}\rho)}{Nf} = \frac{\mu_s}{\omega^2 r} \equiv s$$

BMS COLLEGE OF ENGINEERING, BANGALORE-19
DEPARTMENT OF BIOTECHNOLOGY
Faculty : Dr. V. Saisha

Since both $m(1 - v \cdot \rho)$ and f are inherent properties of the particle and solvent, respectively, s is a constant for any given particle/solvent. The range of values observed in biochemistry varies widely from as low as 1×10^{-13} s for a protein to $10\,000 \times 10^{-13}$ s for intracellular organelles. A more useful notation is provided by the Svedberg, S, with one Svedberg equivalent to 1×10^{-13} s. Factors affecting 's' are mol .wt., frictional force and solute .

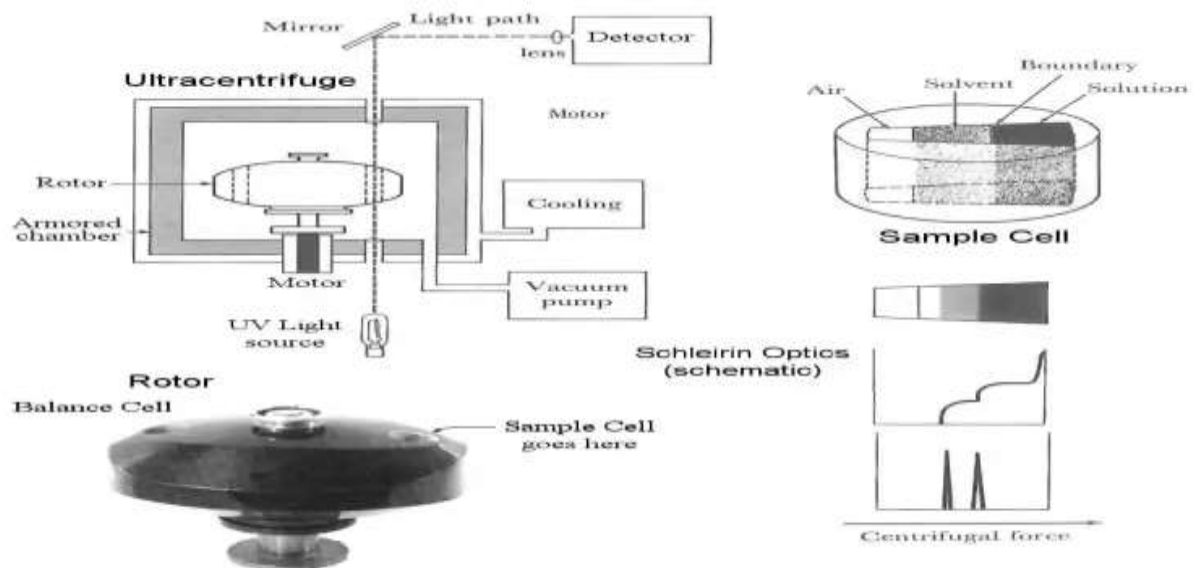
Types of ultracentrifugation :

In ultracentrifugation, analytes are separated on the basis of their sedimentation when they experience a centripetal force. An ultracentrifuge is a centrifuge specially designed to spin at an extremely high rate of speed. Some ultracentrifuges can exert forces as much as 1 million times that of gravity. The physics of sedimentation shows that the sedimentation rate of a particle or molecule depends on several things, including the force, the density of the fluid, and the size and density (or concentration) of the particles in the fluid. Applying a force stronger than gravity can increase the sedimentation rate. It can also magnify differences in sedimentation behavior between different molecules. This makes ultracentrifugation a convenient technique for separating molecules of different sizes. Ultracentrifugation is used as both a *preparative* and an *analytical* technique. For example, the ultracentrifuge is commonly used to isolate samples of pure DNA

Analytical centrifuge	Preparative
Uses small sample size	Large sample size can be used.
Built in optical system for analysis of progression of mol. During centrifugation	No optical read-out – collect fractions and analyze them after the run
Uses relatively pure sample	Less pure sample can be used
Determination of sedimentation co-efficient ., MW of molecules	Can be used to estimate sedimentation coefficient and MW Generally used to separate organelles and molecules

BMS COLLEGE OF ENGINEERING, BANGALORE-19
DEPARTMENT OF BIOTECHNOLOGY
Faculty : Dr. V. Saisha

Instrumentation of Ultracentrifugation



- Components
 1. drive and speed control
 2. temperature control
 3. vacuum system
 4. rotors

Drive and speed control

The rotor is placed on a spindle attached to a motor below the centrifuge. The chamber is surrounded with thick steel armour to contain the fragments in the event of a rotor failure

Vacuum system

The rotor chamber is evacuated with a vacuum pump to reduce air friction and cooled with a cooling jacket to maintain constant temperature and prevent overheating

Types of rotor

BMS COLLEGE OF ENGINEERING, BANGALORE-19
DEPARTMENT OF BIOTECHNOLOGY
Faculty : Dr. V. Saisha

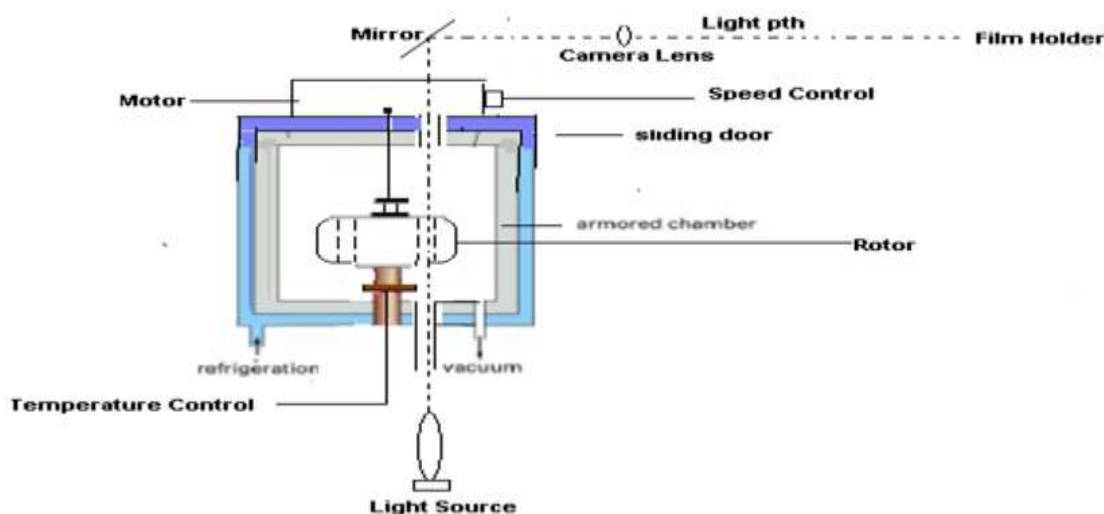
Preparatory ultracentrifuges are available with wide variety of rotors

Fixed angle rotor-made of single block of metal and hold tubes in cavities bored at predetermined angles.

Vertical tube rotor

Swing bucket rotors allow the tubes to hang on hinges so that the tubes reorient to the horizontal as the rotor accelerates

Outline design of an analytical ultracentrifuge.



Sample is placed in a sample cell in a rotor contained in an evacuated and refrigerated chamber. Centrifugation is carried out at very high speed during which the behavior of the sample is analyzed. The cell is scanned with parallel light as it passes through the detection system on each revolution. Transparent windows at the top and bottom of sample cells allow light to pass through the sample. Optical systems use absorbance, fluorescence or refractive index to detect sample concentration as a function of position in the cell. Refractive index measurements of sample is continuously compared to that of solvent alone in a double-sector sample cell. This data is continuously collected during the experiment, stored in a microcomputer and subsequently analyzed. Analytical ultracentrifuges achieve relative centrifugal forces of up to 600 000 g and facilitate analysis of the behavior of sample particles during the centrifugation experiment.

Applications

BMS COLLEGE OF ENGINEERING, BANGALORE-19
DEPARTMENT OF BIOTECHNOLOGY
Faculty : Dr. V. Saisha

Determine sample purity

Characterize assembly and disassembly mechanisms of bimolecular complexes

Determine subunit stoichiometry

Detect and characterize macromolecular conformational changes

Study the behavior of bio macromolecules under a wide range of solvent conditions where they will not interact with support materials

Sedimentation analysis allows behavior of macromolecules across a wide concentration range to be studied in a single experiment; samples in a size-range up to tens of millions of daltons which are difficult to study by other methods (e.g. viruses) are amenable to sedimentation analysis.

In current practice, two distinct types of analytical centrifugation experiment are commonly performed:

Sedimentation Velocity Analysis

Sedimentation velocity analysis involves following the behavior of sample particles as they move through the solvent during sedimentation at relatively high centrifugal speeds. Particles become distributed in a concentration gradient of characteristic shape which changes during the experiment. At the beginning of the experiment, sample is distributed uniformly through the sample cell. The lamella of molecules at the interface between this solution and air is referred to as the boundary. During centrifugation, these molecules move towards the bottom of the sample

cell which causes a change in the boundary position and shape which may be followed by absorbance, fluorescence or refractive index. This is because sedimentation forces the sample particles to move through the solvent away from the centre of rotation while diffusion causes spreading of the boundary resulting in a change in boundary shape during the experiment. Computer methods for analysis of these boundaries have been greatly improved in recent years making a number of physical and chemical parameters accessible to determination by this method. These include the buoyant mass [$m(1 - v \cdot \rho)$], sedimentation and diffusion coefficients of individual molecular species

Sedimentation equilibrium analysis

The sedimentation conditions for sedimentation equilibrium analysis differ from those used in sedimentation velocity in that much lower centrifugal speeds are used in much shorter columns of solvent. Equilibrium is formed between sedimentation and diffusion which results in

BMS COLLEGE OF ENGINEERING, BANGALORE-19
DEPARTMENT OF BIOTECHNOLOGY
Faculty : Dr. V. Saisha

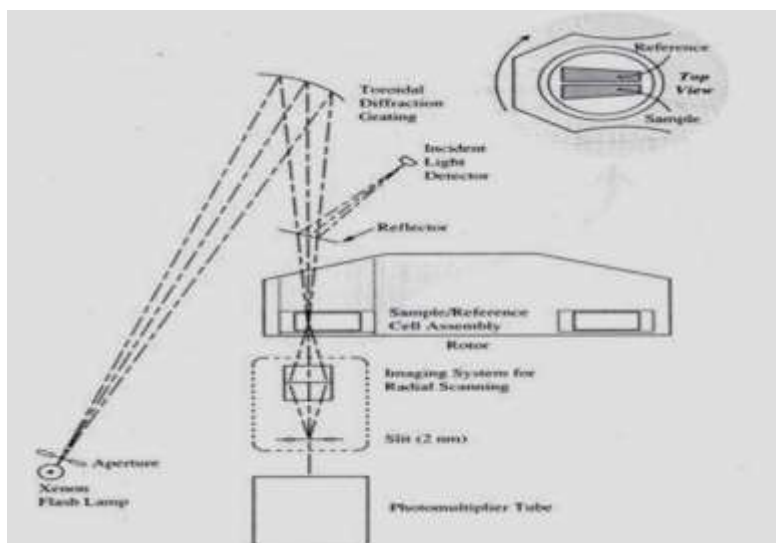
formation of smooth concentration gradient rather than fixed boundaries. Because formations of a stable equilibrium is quite slow, sedimentation equilibrium experiments may require days of centrifugation making them also much slower than sedimentation velocity.

Methods of Detection and Data Collection

- The essential data obtained from an experiment with the analytical ultracentrifuge is a record of the concentration distribution. The most direct means of data collection is a set of concentration measurements at different radial positions and at a given time. This is approached most closely by methods of detection that measure the absorbance of the sample at a given wavelength at fixed positions in the cell; for solutes obeying the BeerLambert law, the absorbance is proportional to concentration

Methods used :

- Refractometric methods
- Rayleigh interference optics
- Absorbance



Schematic diagram of the Analytical Ultracentrifuge

Preparative ultracentrifuges

- It is used for isolation of molecules.
- Determination of relative molecular mass

Types of centrifugal separation

- Differential separation
- Density Gradient separation

BMS COLLEGE OF ENGINEERING, BANGALORE-19
DEPARTMENT OF BIOTECHNOLOGY
Faculty : Dr. V. Saisha

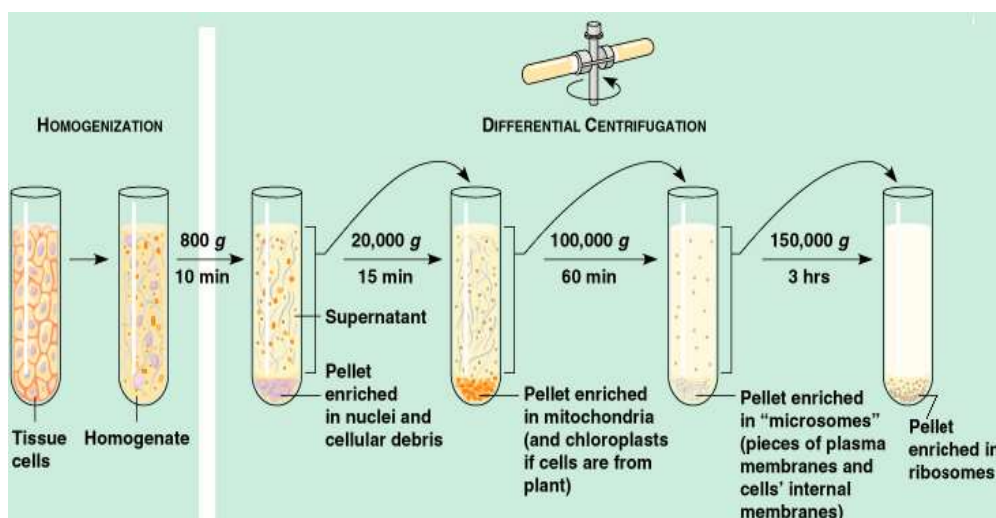
Differential Centrifugation

- Introduced by Albert Claude
- Isolation of subcellular fractions by increasing RCF.(relative centrifugal force)
- Isolated organelles are separated on the basis of their weight
- The 'suspension of isolated organelles is spun for different combinations of speed and time The lowest speed and shortest time separates out the heaviest organelles with high speeds and longer times separating the lightest organelles
- Differential centrifugation – pelleting Start with a uniform mixture of sample – molecules or organelles in buffer--- Centrifuge to obtain pellet of heavier molecules, and supernatant

Because all sized particles are evenly distributed in the beginning, the pellet will contain primarily large sized particles but will also contain some of the other sized particles.Re- suspend the pellet and re-centrifuge (2-3 times) to get fairly pure prep of heaviest particles, but 100% separation is not possible by this method. Can take supernatant and re-centrifuge to pellet the next largest particle at increasingly higher centrifugal forces can largest particle at increasingly higher centrifugal forces can partially purify the different sized particles.

To obtain good separation, size and density of the particles must differ by several orders of magnitude. Commonly used for separation of organelles

Usually fixed angle rotor is used.



BMS COLLEGE OF ENGINEERING, BANGALORE-19
DEPARTMENT OF BIOTECHNOLOGY
Faculty : Dr. V. Saisha

Density gradient centrifugation: Rate zonal (also termed sedimentation velocity, zone centrifugation)

Uses a continuous density gradient of solvent such as sucrose. Density increases toward the bottom of tube. Sample is layered on top. Centrifuge until molecules form discrete bands depending upon their sedimentation values. Generally stop the run before any of the molecules reaches the bottom. 100% separation is possible with a small sample

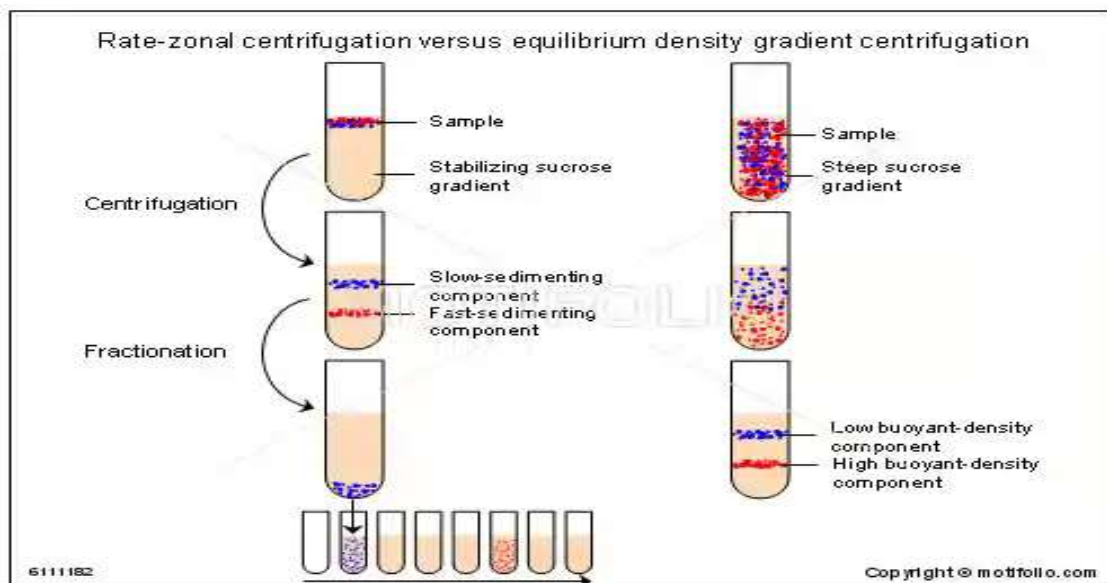
Separates molecules based on size (MW).

Used to separate various types of macromolecules – mixture of proteins; different types of RNA; separate DNA from RNA, or proteins; ribosomal subunits; polyribosomes

Swinging bucket rotor is always used

Isopycnic uses a gradient of CsCl to separate based on buoyant densities. More dense components will equilibrate in more dense region of CsCl and less dense components will equilibrate in the less dense region

Rate zonal separates components based on their S-value, which determines how quickly the particle under investigation will move through sucrose gradient



- Application

Separation of biological molecules ie, biomolecules on basis of density like DNA, RNA.

Separation of subcellular organelles-Chloroplast

Separation of labelled and non labelled molecules.

Separation of ribosomal subunit given as Svedberg units

BMS COLLEGE OF ENGINEERING, BANGALORE-19
DEPARTMENT OF BIOTECHNOLOGY
Faculty : Dr. V. Saisha

VISCOMETRY

The size and shape of a molecule affect its ability to move in a fluid solution. Its properties also alter the bulk properties (physical properties) of a fluid.

Several techniques can be used for the estimation of molecular weight and shape and can be grouped into: 1.translational motion which is shown by Brownian movt. of molecules in absence of an external force ., and on application of an external force.eg. Sedimentation, ultracentrifugation etc., 2. Rotational motion - techniques like EPR, NMR , birefringence etc

Viscosity

Viscosity is defined as the force needed to maintain a velocity gradient dv/dx in a fluid, normal to the applied force. It is the measurement of overall properties of a solution. It is marked affected by translational and rotational motion of dissolved large molecules.

- Mathematically given as :

$F = \eta A (dv/dx)$ Where A = area of fluid parallel to flow ., η = co-efficient of viscosity .,
 dv/dx = velocity gradient /shear

Frictional properties of macromolecules in solution

Motion in a solution is comparatively slower than in a gas phase due to the frictional forces between molecules in a condensed phase. Consider the external force F is applied to a molecule of mass m ., then the equation for motion in vacuum is given as $F=ma$, $a=dv/dt$, v is velocity. However in a real fluid, the molecule is subjected to frictional drag /resistance .This drag is proportional to the velocity as long as the motion is not fast to cause turbulence. Then the equation of motion (translational motion) given as: $F-fv = m dv/dt$., where fv =frictional force opposing the applied force, f -translational frictional co-efficient and depends on the nature of fluid.

The linear differential equation for motion in a fluid can be given as:

- $V(t) = F/f + [v_0 - F/f] e^{-ft/m}$ v_0 – velocity at $t=0$ parallel to applied force., F – constant

Three important consequences can be realized .viz.,

BMS COLLEGE OF ENGINEERING, BANGALORE-19
DEPARTMENT OF BIOTECHNOLOGY
Faculty : Dr. V. Saisha

1. The particle is accelerated for a short time after external force is applied.
2. The velocity decays exponentially from initial value v_0 to final value
3. The final velocity $V(\alpha) = F/f$ and is linear to applied force.

Frictional force arises in a fluid because of attraction among molecules of a fluid. (Cohesive forces and adhesive forces). Therefore the movement of a solid particle in a fluid causes disturbance in the movement of solvent particles. The molecules nearest to the solid particle are the most perturbed and this disturbance decreases as one moves away from the particle. .

To calculate the frictional force, the force required to maintain their disturbed condition (vel. Distribution of solvent molecules) is to be calculated. This is related to properties of fluid called viscosity.

Consider a fluid bound by two surfaces, the force required to slide one surface over another is proportional to the area of sheet, and reflects the differences in velocity. Consider the surfaces as sheets of fluid, for small velocities increments then

$F = \eta A (dv/dx)$ Where A = area of fluid parallel to flow., η = proportionality co-efficient called as coefficient of viscosity and depends on the properties of fluid. ., dv/dx = velocity gradient called as shear.

Measurement of viscosity

The simplest equipment used is a capillary viscometer.

Principle: the hydrostatic pressure provides the force .and is given as: $F = P\pi a^2$., where
 a – radius of capillary.

The differential force on a cylindrical sheet of fluid at a radial distance of x is given as

$$dF = 2\pi x P dx \text{ ., cross-section area - } 2\pi x dx \text{ .} \quad \text{-----1}$$

If the fluid is flowing through a capillary at steady rate, this force should be balanced by frictional force .The frictional force which is given as

$$F = -\eta 2\pi x l (dv/dx) \text{ ., where the lateral surface of a cylindrical sheet} = 2\pi x l$$

BMS COLLEGE OF ENGINEERING, BANGALORE-19
DEPARTMENT OF BIOTECHNOLOGY
Faculty : Dr. V. Saisha

Therefore the net force on the fluid sheet equals the differential forces felt by the two sides of a sheet. Which is given as

$$dF = -\eta l 2\pi \left[d\left(x \frac{dv}{dx}\right) / dx \right] dx \quad \text{-----2}$$

Equating the equations 1&2.,

$$2\pi x P dx = -\eta l 2\pi \left[d\left(x \frac{dv}{dx}\right) / dx \right] dx \text{ then },$$

$$Px = -\eta l \left\{ d\left[x \left(\frac{dv}{dx}\right)\right] / dx \right\} \quad \text{-----3}$$

On integration.,

$Px^2 / 4 + C_1 = -\eta l x \left(\frac{dv}{dx} \right)$., where C is an integration constant. Dividing through by x and integrating we get

$$Px^2 / 4 + C_1 \ln x + C_2 = -\eta / v \quad C_1 \text{ and } C_2 \text{ are evaluated from boundary conditions.}$$

At the center of a capillary $x=0$, velocity of flow is finite. Therefore $C_1=0$

At the outside velocity $=0$ (stick boundary conditions are taken) Therefore $C_2 = -Pa^2/4$

The velocity profile of a fluid in a capillary will have a parabolic shape and the flow velocity (v) is given as:

$$v = (P/4 \eta l)(a^2 - x^2) \quad v = (P/4 \eta l)(a^2 - x^2)$$

However, easily measurable quantity is vol. rate of flow which is given as the vel. of each cylindrical sheet X area ($2\pi x P dx$) and integrated from centre of capillary to radius. Representing the above equation with respect to vol. rate of flow we get the equation :

$$\begin{aligned} dv/dt &= \int_0^a 2\pi x v dx = \frac{xP}{2\eta l} \int_0^a (a^2 - x^2) x dx \\ &= \pi Pa^4 / 8 \eta l \end{aligned}$$

This equation is called the Poiseuille's equation

Dependence of Viscosity on Characteristics of Solute

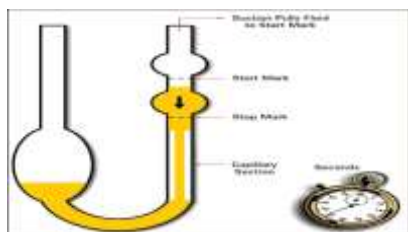
The relationship between $[\eta]$ and molecular mass and shape is complex. However, generally speaking, the larger the molecule and the more extended its shape, the greater the value of $[\eta]$.

BMS COLLEGE OF ENGINEERING, BANGALORE-19
DEPARTMENT OF BIOTECHNOLOGY
Faculty : Dr. V. Saisha

An important factor in overall shape is the axial ratio (i.e. the ratio of the longest to the shortest axis for the molecule). Fibrous molecules have larger axial ratios than globular molecules of the same mass and therefore give more viscous solutions. For double-stranded DNA the relationship between mass and $[\eta]$ has been empirically determined as; $\log([\eta] + 5) = 0.665 \cdot \log M - 2.863$, while for random coil proteins of n residues dissolved in 6Nguanidinium hydrochloride it is; $[\eta] = 0.716 \cdot n^{0.66}$

Changes in viscosity may be used to follow processes in which the overall shape or elasticity of the molecule is altered. Examples of this include intercalation of drugs into DNA and protein denaturation

Measurement with a capillary viscometer



In a typical measurement, the fluid of density ρ is allowed to fall from a height h_1 - h_2 and time taken is determined. The hydrostatic pressure provides the force and is given as ρgh , $h = h_2 - h_1$ i.e. the diff. between 2 ends of the column. The pressure decreases as the ht. of fluid in capillary decreases. The time reqd. for total vol. V to flow is obtained by integration of Poiseuille's law

$$t = 8\eta l / \pi g \rho a^4 \int_{h_1}^{h_2} dV / h$$

The integral is a constant for a given apparatus and is found by calibration.

Disadvantages of capillary viscosity measurements

- Large volumes of solution are required.
- Shear forces generated by the flow gradients are large.

Effect of shear on measured velocity

BMS COLLEGE OF ENGINEERING, BANGALORE-19
DEPARTMENT OF BIOTECHNOLOGY
Faculty : Dr. V. Saisha

Shear stress: $S = F/A = \eta (dv/dx)$ (defined as the force per unit area resulting from solution flow. it is the velocity gradient perpendicular to force.) Alters the η of media. Eg. 1) Shear stress causes orientation of long rigid molecules like DNA 2) Causes distortion of coil distribution of flexible molecules. 3) Very high shear stress leads to cleavage of molecules. Therefore measurements have to be made at low shear. Mathematically Average shear stress is given as $S_x = \eta (dv/dx) = -Px/2l$

On integrating, $S = \int_0^a 2\pi x dx S_x = -Pa/3l$ from the above eqn., we can infer that as pressure depends on difference in ht., shear stress can be varied with changes in ht.

Rotating cylinder viscometer

The rotational viscometer (also referred to as a Brookfield viscometer) is a concentric cylinder rheometer. It is used for lower shear stresses. Viscosity is determined from the amount of torque needed to rotate a cylinder (called a spindle) with a known geometry. A rotating magnetic field applies a const. torque on an inner cylinder, which is free floating in soln. In response the inner cylinder rotates with constant angular velocity, which is less than that of the rotating magnetic field. The relative viscosities are given as $\eta_1/\eta_2 = \omega_2/\omega_1$.

Shear can be altered by changing dimensions of inner cylinder or by changing the strength of magnetic field.

Effect of solute molecules on viscosity

The viscosity of solution of particles is a complex function of the concentration. At high conc. the distortion of the vel. pattern of liquid by one particle affects the shear of neighboring particle. The viscosity of solution can be expressed as a power series relative to viscosity of pure solvent. Given as: $\eta = \eta_0 (1 + K_1C_2 + K_2C_2^2 + \dots)$.

The Relative viscosity is given as $\eta/\eta_0 = \eta_{rel}$ and

Specific viscosity $\eta_{sp} = \eta_{rel} - 1 = (K_1C_2 + K_2C_2^2 + \dots)$, K is of interest for determining properties of individual molecules and it is defined by the intrinsic viscosity. $[\eta]$ obtained experimentally.

The intrinsic viscosity given as $[\eta] = \lim_{C_2 \rightarrow 0} \frac{\eta_{sp}}{C_2} = \lim_{C_2 \rightarrow 0} (K_1 + K_2C_2 + \dots)$

Also given as: $[\eta] = 2.5(V_2 + \delta V_1)$, V_2 , V_1 - partial specific volume of solute and water

BMS COLLEGE OF ENGINEERING, BANGALORE-19
DEPARTMENT OF BIOTECHNOLOGY
Faculty : Dr. V. Saisha

δ -hydration gm/gm.

Properties of $[\eta]$

- $[\eta]$ is highly sensitive to shape. It is also represented as $[\eta] = vV_n N_0 / M$ where v = simha factor and gives information on shape based on the axial ratios. For axial ratios > 10 , the simha factor is given by asymptomatic eqns eg.
- $v = (a/b)^2 / 5 [\ln(2a/b) - 1/2] + (a/b)^2 / 15 [\ln(2a/b) - 3/2] + 14/15$ for prolate ellipsoids. a/b - axial ratio.,
- Intrinsic viscosity can be given with respect to molecular wt. as : $[\eta] = KM^\alpha$ this is called as Mark Houwink eqn. For a homologous series of rods of const. dia., the mol. wt. is propotional to length. $\alpha = d[\ln[\eta]]/d[\ln a/b]$. Intrinsic viscosity is also sensitive to conformation., eg. If a globular protein is denatured mol. Wt. remains the same but $[\eta]$ changes. (increases)

Viscoelastic relaxation

- First shown by Lynn Klotz and Bruno Zimm developed a technique to study long DNA. low shear viscometric method.
- Useful for the determination of the molecular weight of large ($>10^{10}$) DNA molecules
- Involves mildly stretching long DNA molecules by hydrodynamic shear forces and then removing the force so that the molecule relaxes back to its normal conformation; the relaxation time is related to, and can be used to determine with accuracy, the molecular weight of DNA molecules of the size found in eukaryotic chromosomes.
- When a torque is applied to DNA soln. the average chain configuration of DNA is altered from a stiff coil to elongated structure. The energy is from the applied torque coupled through shear gradient in fluid. eventually a steady state is reached, DNA mol have some stored energy in their chain confirmation. When the torque is removed, the rotation would slow down and stop if there were no DNA. However, with DNA present, rotor can't stop., until the DNA mol. have relaxed backed to random coil. The motor reverses direction and gradually stops. The force that coupled with the original torque

BMS COLLEGE OF ENGINEERING, BANGALORE-19
DEPARTMENT OF BIOTECHNOLOGY
Faculty : Dr. V. Saisha

exerts torque on the rotor as chains relaxes .this effect can last for a considerable time and is characterized by relaxation time. T_k , $T_k = (M\eta_0[\eta]/RT) 1/\lambda_k$

- λ_k is a function of eigen values obtained when dynamics of relaxation are computed. The equation yields information on Mol. Wt.
- Alternate expression for molecular wt. $M = k[\eta + b]^{3/2}$ $K = 19.8$ for linear DNA $b = 500 \text{ ml/g}$ Neglecting b for large molecules, The eqn. can be written as : **$M = 1.45 \times 10^8 t^{3/5}$**

Protocol

- Cells lysed in rotating cylinder viscometer----- Treatment with detergent and enzymes-----Application of torque-----Removal of torque before one revolution
- The torque generates a subsequent torque on rotor as chains relax. Characterized by relaxation time Mol. Wt. can be determined using the formula **$M = 1.45 \times 10^8 t^{3/5}$**

ELECTRON MICROSCOPY

The electron microscope is a type of microscope that uses a beam of electrons to create an image of the specimen. It is capable of much higher magnifications and has a greater resolving power than a light microscope, allowing it to see much smaller objects in finer detail. All electron microscopes use electromagnetic and/or electrostatic lenses to control the path of electrons. Glass lenses, used in light microscopes, have no effect on the electron beam. The basic design of an electromagnetic lens is a solenoid (a coil of wire around the outside of a tube) through which one can pass a current, thereby inducing an electromagnetic field. The electron beam passes through the centre of such solenoids on its way down the column of the electron microscope towards the sample. Electrons are very sensitive to magnetic fields and can therefore be controlled by changing the current through the lenses. The faster the electrons travel, the shorter their wavelength. The resolving power of a microscope is directly related to the wavelength of the irradiation used to form an image. Reducing wavelength increases resolution. Therefore, the resolution of the microscope is increased if the accelerating voltage of the electron beam is increased. The accelerating voltage of the beam is quoted in kilovolts (kV). The relationship between the limit of resolution and the wavelength of the illuminating radiation holds true for any form of radiation, whether it is a beam of light or a beam of electrons. With electrons, however, the limit of resolution can be made very small and is given by Abbe's equation $d = 0.61 \lambda / NA$., $NA = n \cdot \sin \alpha$

Resolution of electron microscopes is $\lambda = h (2meV)^{-1/2}$., $m = \text{mass}$., $e = \text{charge}$., $V = \text{accelerating voltage}$. In case of EM: $d = 0.753 / \alpha \cdot \sqrt{V}$, nm $d = \text{resolution in nm}$., $\alpha = \text{half opening angle of objective (in radians)}$., $V = \text{accelerating voltage}$., $d (100 \text{ kV}) = 0.24 \text{ nm}$ $\alpha \approx 0.01 \text{ radians}$ The higher the energy of the electrons, the lower the wavelength, the higher the resolution. The wavelength of an electron decreases as its velocity increases. In an electron microscope with an accelerating voltage of 100,000 V, the wavelength of an electron is 0.004 nm. In theory the resolution of such a microscope should be about 0.002 nm, which is 10,000 times greater than that of the light microscope. Because the aberrations of an electron lens are considerably harder to correct those of a glass lens, however, the practical resolving power of most modern electron microscopes is, at best, 0.1 nm (1 Å) Furthermore, problems of specimen preparation, contrast, and radiation damage effectively limit the normal resolution for biological objects to 2 nm (20 Å). This is nonetheless about 100 times better than the resolution of the light microscope. The resolution in Transmission electron microscopes (TEM): 40 – 1200 kV and Scanning electron microscopes (SEM): 1 – 30 kV Effective instrument resolution is TEM: $\sim 0.1 \text{ nm}$., SEM: $\sim 1 \text{ nm}$

Sample Preparation

Materials to be viewed in an electron microscope generally require processing to produce a suitable sample. This is mainly because the whole of the inside of an electron microscope is under high vacuum in order to enable the electron beam to travel in straight lines. The

BMS COLLEGE OF ENGINEERING, BANGALORE-19
DEPARTMENT OF BIOTECHNOLOGY
Faculty : Dr. V. Saisha

technique required varies depending on the specimen, the analysis required and the type of microscope:

Cryofixation - freezing a specimen rapidly, typically to liquid nitrogen temperatures or below, that the water forms ice. This preserves the specimen in a snapshot of its solution state with the minimal of artefacts. An entire field called cryo-electron microscopy has branched from this technique. With the development of cryo-electron microscopy, it is now possible to observe virtually any biological specimen close to its native state.

Fixation - a general term used to describe the process of preserving a sample at a moment in time and to prevent further deterioration so that it appears as close as possible to what it would be like in the living state, although it is now dead. In chemical fixation for electron microscopy, glutaraldehyde is often used to crosslink protein molecules and osmium tetroxide to preserve lipids.

Dehydration - removing water from the samples. The water is generally replaced with organic solvents such as ethanol or acetone as a stepping stone towards total drying for SEM specimens or infiltration with resin and subsequent embedding for TEM specimens.

Embedding - infiltration of the tissue with wax (for light microscopy) or a resin (for electron microscopy) such as araldite or LR White, which can then be polymerised into a hardened block for subsequent sectioning.

Sectioning - the production of thin slices of the specimen. For light microscopy, the sections can be a few micrometres thick but for electron microscopy they must be very thin so that they are semitransparent to electrons, typically around 90nm. These ultra-thin sections for electron microscopy are cut on an ultramicrotome with a glass or diamond knife. Glass knives can easily be made in the laboratory and are much cheaper than diamond, but they blunt very quickly and therefore need replacing frequently.

Staining - uses heavy metals such as lead and uranium to scatter imaging electrons and thus give contrast between different structures, since many (especially biological) materials are nearly "transparent" to the electron beam. By staining the samples with heavy metals, we add electron density to it which results in there being more interactions between the electrons in the primary beam and those of the sample, which in turn provides us with contrast in the resultant image. In biology, specimens can be stained "en bloc" before embedding and/or later, directly after sectioning, by brief exposure of the sections to solutions of the heavy metal stains.

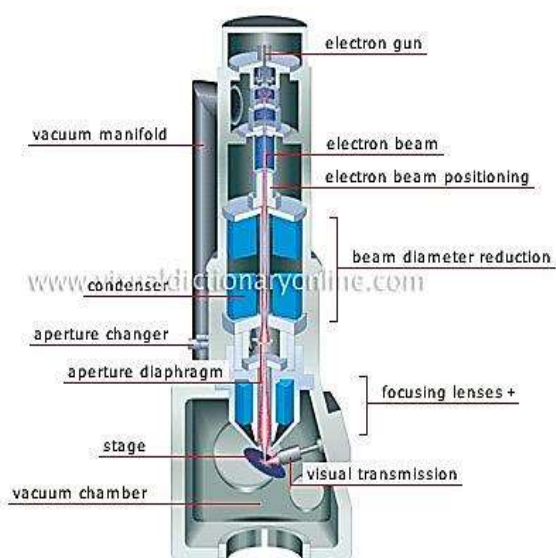
Freeze-fracture and freeze-etch - a preparation method particularly useful for examining lipid membranes and their incorporated proteins in "face on" view. The fresh tissue or cell suspension is frozen rapidly (cryofixed), then fractured by simply breaking or by using a microtome while maintained at liquid nitrogen temperature. The cold, fractured surface is generally "etched" by increasing the temperature to about -95°C for a few minutes to let some

BMS COLLEGE OF ENGINEERING, BANGALORE-19
DEPARTMENT OF BIOTECHNOLOGY
Faculty : Dr. V. Saisha

surface ice sublime to reveal microscopic details. For using SEM, the sample is now ready for imaging. For the TEM, it can then be rotary-shadowed with evaporated platinum at low angle (typically about 6°) in a high vacuum evaporator. A second coat of carbon, evaporated perpendicular to the average surface plane is generally performed to improve stability of the replica coating. The specimen is returned to room temperature and pressure, and then the extremely fragile "shadowed" metal replica of the fracture surface is released from the underlying biological material by careful chemical digestion with acids, hypochlorite solution or SDS detergent. The floating replica is thoroughly washed from residual chemicals, carefully picked up on an EM grid, dried then viewed in the TEM.

Sputter Coating - an ultra-thin coating of electrically-conducting material, deposited by low vacuum coating of the sample. This is done to prevent charging of the specimen which would occur because of the accumulation of static electric fields due to the electron irradiation required during imaging. It also increases the amount of secondary electrons that can be detected from the surface of the sample in the SEM and therefore increases the signal to noise ratio. Such coatings include gold, gold/palladium, platinum, chromium etc.

The electron microscope -working



1. Electron optical column consists of:– electron gun -electron source to produce electrons.,– magnetic lenses to de-magnify the beam– magnetic coils to control and modify the beam.,– apertures to define the beam, prevent electron spray,etc.
2. Vacuum systems consists of:– chamber which “holds” vacuum, pumps to produce vacuum– valves to control vacuum, gauges to monitor vacuum
3. Signal Detection & Display consists of:– detectors which collect the signal– electronics which produce an image from the signal

BMS COLLEGE OF ENGINEERING, BANGALORE-19
DEPARTMENT OF BIOTECHNOLOGY
Faculty : Dr. V. Saisha

Disadvantages of Electron Microscopy

Electron microscopes are very expensive to buy and maintain. They are dynamic rather than static in their operation: requiring extremely stable high voltage supplies, extremely stable currents to each electromagnetic coil/lens, continuously-pumped high/ultra-high vacuum systems and a cooling water supply circulation through the lenses and pumps. As they are very sensitive to vibration and external magnetic fields, microscopes aimed at achieving high resolutions must be housed in buildings with special services.

A significant amount of training is required in order to operate an electron microscope successfully and electron microscopy is considered a specialised skill.

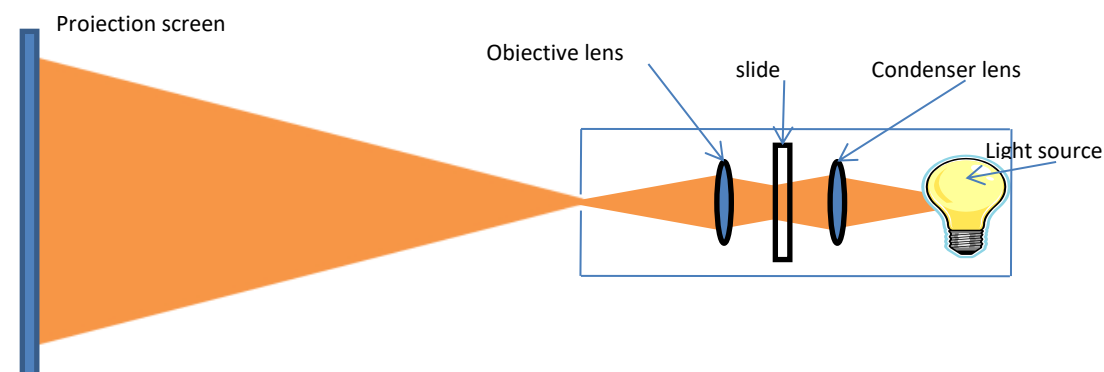
The samples have to be viewed in a vacuum, as the molecules that make up air would scatter the electrons. This means that the samples need to be specially prepared by sometimes lengthy and difficult techniques to withstand the environment inside an electron microscope. Recent advances have allowed some hydrated samples to be imaged using an environmental scanning electron microscope, but the applications for this type of imaging are still limited.

There are several types of electron microscopy. The two most common types are transmission electron microscopy and scanning electron microscopy

TRANSMISSION ELECTRON MICROSCOPE (TEM)

Transmission electron microscopy (TEM) involves a high voltage electron beam emitted by a cathode and formed by magnetic lenses. The electron beam that has been partially transmitted through the very thin (and so semitransparent for electrons) specimen carries information about the structure of the specimen. The spatial variation in this information (the "image") is then magnified by a series of magnetic lenses until it is recorded by hitting a fluorescent screen, photographic plate, or light sensitive sensor such as a CCD (charge-coupled device) camera. The image detected by the CCD may be displayed in real time on a monitor or computer. Simplistically, In its operation a TEM can be thought of as analogous to a slide projector

Slide projector



TEM



BMS COLLEGE OF ENGINEERING, BANGALORE-19
DEPARTMENT OF BIOTECHNOLOGY
Faculty : Dr. V. Saisha

In a slide projector white light from the bulb is focussed onto the slide by a condenser lens. This produces an image which is focussed by the objective lens to a point (crossover). The image is then projected onto a screen and magnified by varying the distance from the crossover to the screen. In a TEM the electron beam is focussed on the sample using the condenser lens system. This produces an image which is focussed by the objective lens to a point (crossover). This image is then magnified by a series of projector lenses to vary the size of the image on a fluorescent screen. Changing the current of an electromagnetic lens alters its focal length altering magnification. Transmission electron microscopes produce two-dimensional, black and white images.

Resolution of the TEM is limited by spherical and chromatic aberration, but a new generation of aberration correctors has been able to overcome or limit these aberrations. Software correction of spherical aberration has allowed the production of images with sufficient resolution to show carbon atoms in diamond separated by only 0.089 nm and atoms in silicon at 0.078 nm at magnifications of 50 million times. The ability to determine the positions of atoms within materials has made the TEM an indispensable tool for nano-technologies research and development in many fields, including heterogeneous catalysis and the development of semiconductor devices for electronics and photonics. In the life sciences, it is still mainly the specimen preparation which limits the resolution of what we can see in the electron microscope, rather than the microscope itself.

In overall design the transmission electron microscope (TEM) is similar to a light microscope. The source of illumination is a filament or cathode that emits electrons at the top of a cylindrical column about 2 meters high. Since electrons are scattered by collisions with air molecules, air must first be pumped out of the column to create a vacuum. The electrons are then accelerated from the filament by a nearby anode and allowed to pass through a tiny hole to form an electron beam that travels down the column. Magnetic coils placed at intervals along the column focus the electron beam, just as glass lenses focus the light in a light microscope. The specimen is put into the vacuum, through an airlock, into the path of the electron beam. As in the case of light microscopy, the specimen is usually stained, in this case with electron-dense material. Some of the electrons passing through the specimen are scattered by structures stained with the electron-dense material; the remainder are focused to form an image - in a manner analogous to the way an image is formed in a light microscope - either on a photographic plate or on a phosphorescent screen. Because the scattered electrons are lost from the beam, the dense regions of the specimen show up in the image as areas of reduced electron flux, which look dark.

BMS COLLEGE OF ENGINEERING, BANGALORE-19
DEPARTMENT OF BIOTECHNOLOGY
Faculty : Dr. V. Saisha

Main components of a TEM

The Gun., The condenser system., The sample

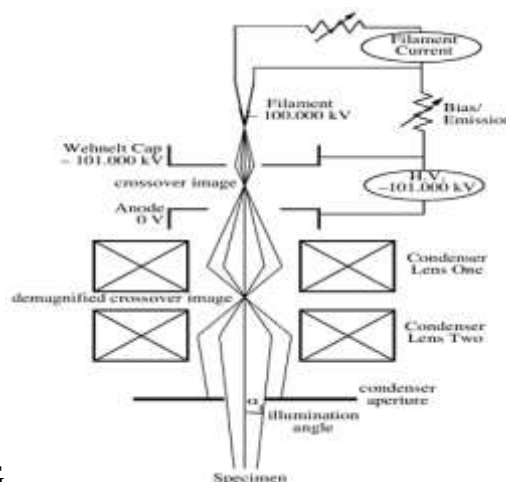
components for Image formation, • Projection of the image (magnification), • Viewing and recording the image

Electron Guns

Two main types— Thermionic and Field Emission. • Thermionic sources produce electrons when heated. • Field emission sources produce electrons when exposed to an intense electric field. FEG's give much more brightness than thermionic systems. • FEG's give a more monochromatic electron source and finer probe (i.e. better resolution).

Field Emission Gun

The tip of a tungsten needle is made very sharp (radius $< 0.1 \mu\text{m}$). • The electric field at the tip is very strong ($> 10^7 \text{ V/cm}$) due to the sharp point effect. • Electrons are pulled out from the tip by the strong electric field applied externally. • Ultra-high vacuum (better than $7.5 \times 10^{-9} \text{ Torr}$ - $6 \times 10^{-6} \text{ Pa}$) is needed to avoid ion bombardment to the tip from the residual gas. • Electron probe diameter $< 1 \text{ nm}$ is possible



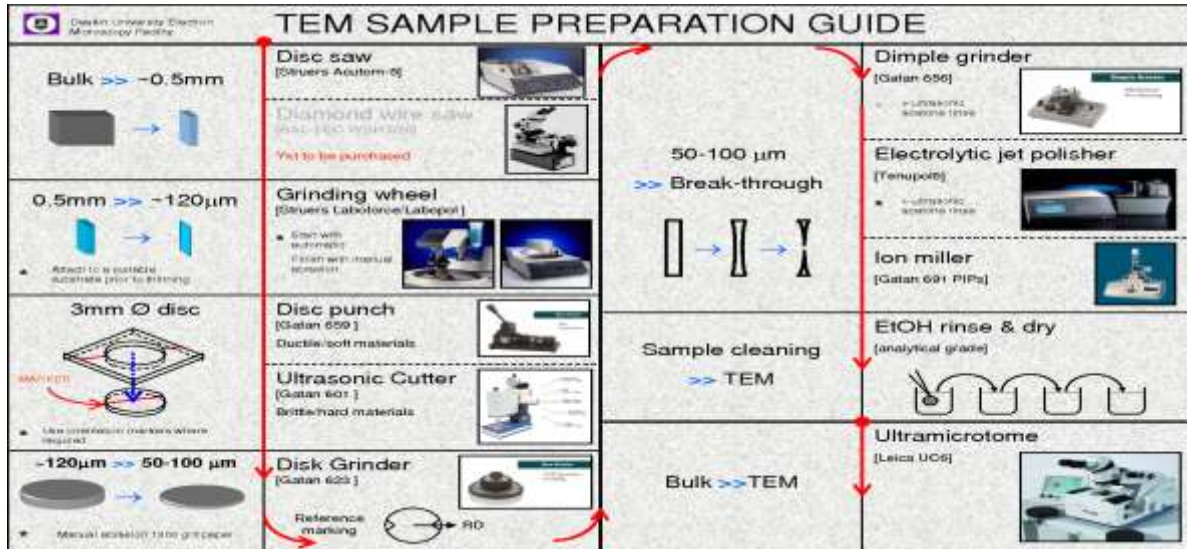
The Condenser System in FEG

The Wehnelt (or 2nd anode in a FEG) focuses the beam to a crossover which is accelerated down the column. • The first condenser de-magnifies the crossover to give a smaller point source – this is referred to as C₁ or spot size. The second condenser lens (C₂) is used to either converge or spread the beam of illumination on the sample. • A condenser aperture is placed in the beam path to remove electrons far from the optic axis which would reduce resolution. The smaller the aperture the better the resolution, but there is an associated decrease in brightness – need to compromise

BMS COLLEGE OF ENGINEERING, BANGALORE-19
DEPARTMENT OF BIOTECHNOLOGY
Faculty : Dr. V. Saisha

The Sample

•Samples are typically 3mm in diameter and <80µm thick. •Sample preparation is tricky and time consuming. sample preparation guide for TEM is given below.



Biological samples require fixing and embedding before being stained with heavy metals (e.g. OsO₄) for contrast prior to ultra microtome sectioning.

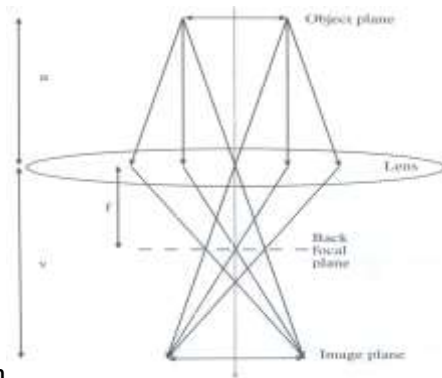
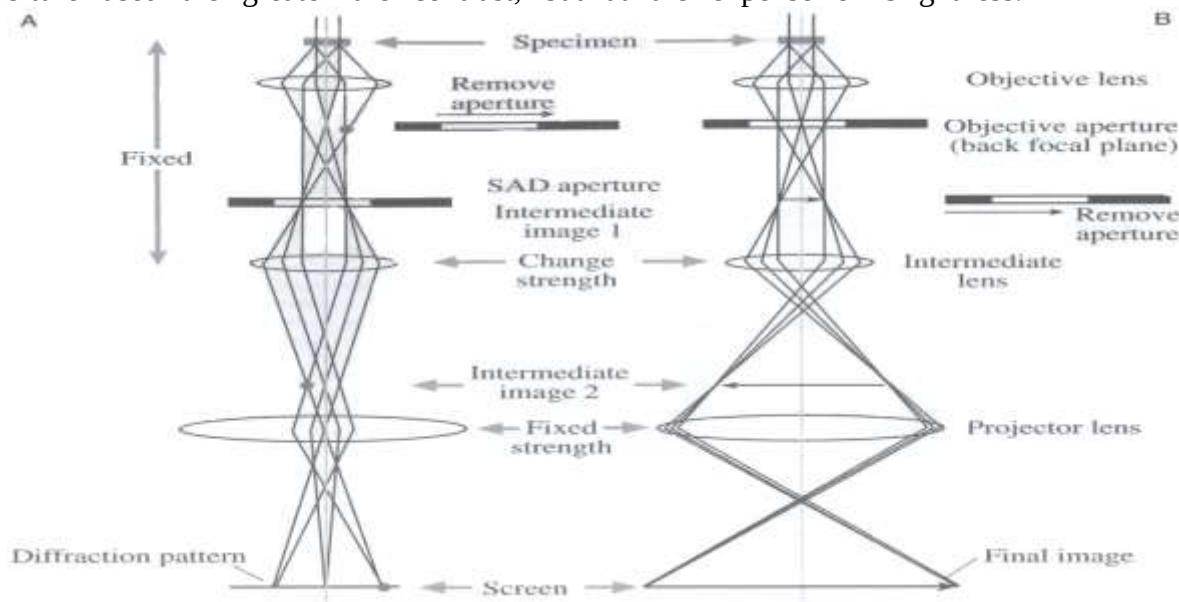


Image Formation

All rays from a point in the object are gathered by the lens and converge to a point in the image. All parallel rays are focused in the focal plane. The back focal plane of the objective lens contains groupings of rays that have left the object at the same angle. The back focal plane contains the diffraction pattern of the sample. Diffraction pattern and image are both formed in the imaging process. The intermediate lens is then focused on either the image plane (for the image), or the back focal plane (for the diffraction pattern).

Imaging Modes Two principle modes of TEM operation, A – Projecting the diffraction pattern, B – Projecting the image. The intermediate lens selects either the Back

Focal Plane or the image plane of the objective lens. Conventionally, in order to get contrast in an image an objective aperture is placed in the beam path at the back focal plane of the objective lens. Any electrons which are scattered by interaction with the sample are prevented from recombining in the image by the aperture. Any regions where electrons are scattered will appear dark in the image. The smaller the aperture used the greater the contrast, but at the expense of brightness.



Contrast formation in TEM

- Absorption of electrons., Scattering of electrons., Diffraction and phase contrast

NOTE: All mechanisms occur at the same time (superposition)

Projection - Magnification

A series of projector lenses are then used to magnify the image formed by the intermediate lens onto a viewing screen. Changing the current in the windings of the lens changes the magnetic field and effectively changes the focal length of the lens. • Increase the current and the focal length (f) of the lens decreases, so a weaker lens (f_1) gives a higher magnification than a stronger lens (f_2) as image distance v increases, but the object distance is unchanged.

Viewing/Recording Images

The image, comprising of a beam of focused electrons falls onto a fluorescent screen therefore producing a visible image. This image can be recorded onto photographic film using the camera located underneath the viewing screen. Modern machines can capture images digitally using CCD cameras. The signal from the beam can be output to external analysis equipment, e.g. STEM detectors, EELS etc. for further analysis

Applications

One of the main strengths of TEM is the ability to obtain diffraction (structural information) and images from the same area at the same time

Differences between TEM and Optical Microscope

	TEM	TOM
Source	100-400 kV electron gun high current densities:	Light source

BMS COLLEGE OF ENGINEERING, BANGALORE-19
DEPARTMENT OF BIOTECHNOLOGY
Faculty : Dr. V. Saisha

	5 x 10 ⁴ Am ⁻² for tungsten filament 1 x 10 ⁶ Am ⁻² for field-emission source	
Condenser lens	Electromagnetic lens, focus adjusted by controlling the lens current	Glass lens, focus adjusted by lens position
Specimen stage	Allows for specimen tilt as well as some z-adjustment	Allows for specimen tilt as well as some z-adjustment
Objective lens	Fine focusing of the image by adjusting the lens current	Fine focusing by adjusting the position of the specimen and the objective lens
Final imaging system	Employs electromagnetic lenses to produce image on a fluorescent screen	Eye piece forming image for direct viewing
Recording system	Computer monitor or TV	Normal viewing or photographic films
Experimental set-up in	Vacuum, better than 10 ⁻⁶ torr	Air, at atmospheric pressure

SCANNING ELECTRON MICROSCOPE (SEM)

Unlike the TEM, where the electrons in the primary beam are transmitted through the sample, the Scanning Electron Microscope (SEM) produces images by detecting secondary electrons which are emitted from the surface due to excitation by the primary electron beam. In the SEM, the electron beam is scanned across the surface of the sample in a raster pattern, with detectors building up an image by mapping the detected signals with beam position.

Because the SEM image relies on electron interactions at the surface rather than transmission it is able to image bulk samples and has a much greater depth of view, and so can produce images that are a good representation of the 3D structure of the sample. SEM images are therefore considered to provide us with 3D, topographical information about the sample surface but will still always be only in black and white.

In the SEM, we use much lower accelerating voltages to prevent beam penetration into the sample since what we require is generation of the secondary electrons from the true surface structure of a sample. Therefore, it is common to use low KV, in the range 1-5kV for biological samples, even though our SEMs are capable of up to 30 kV.

BMS COLLEGE OF ENGINEERING, BANGALORE-19
DEPARTMENT OF BIOTECHNOLOGY
Faculty : Dr. V. Saisha

Main components of a SEM : The Gun ,•The condenser system.,•The sampleImage formation.,•Projection of the image (magnification)…,•Viewing and recording the image.

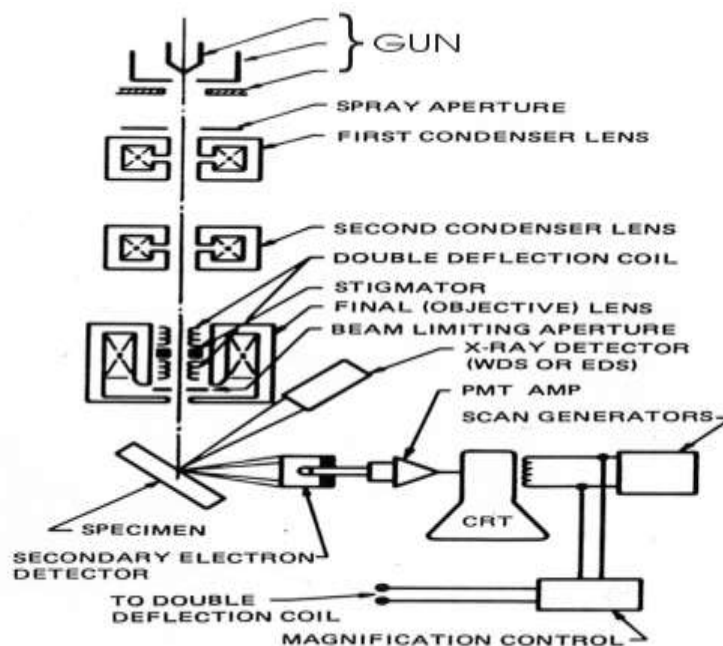


Figure 1.11. Schematic drawing of the electron and x-ray optics of a combined SEM-EPMA.

Working of SEM

- 1) gun –produces a stream of monochromatic electrons
- 2) The stream is condensed by the first condenser lens (usually controlled by the "coarse probe current knob"). This lens is used to both form the beam and limit the amount of current in the beam. It works in conjunction with the condenser aperture to eliminate the high-angle electrons from the beam.
- 3) The beam is then constricted by the condenser aperture (usually not user selectable), eliminating some high-angle electrons.
- 4) The second condenser lens forms the electrons into a thin, tight, coherent beam and is usually controlled by the "fine probe current knob".
- 5) A user selectable objective aperture further eliminates high-angle electrons from the beam.
- 6) A set of coils then "scan" or "sweep" the beam in a grid fashion (like a television), dwelling on points for a period of time determined by the scan speed (usually in the microsecond range).
- 7) The final lens, the objective, focuses the scanning beam onto the part of the specimen desired.
- 8) When the beam strikes the sample (and dwells for a few microseconds) interactions occur inside the sample and are detected with various instruments.

BMS COLLEGE OF ENGINEERING, BANGALORE-19
DEPARTMENT OF BIOTECHNOLOGY
Faculty : Dr. V. Saisha

9) Before the beam moves to its next dwell point these instruments count the number of e-interactions and display a pixel on a CRT whose intensity is determined by this number (the more reactions the brighter the pixel).

10) This process is repeated until the grid scan is finished and then repeated, the entire pattern can be scanned 30 times/sec.

Magnifying in scanning electron microscopes

- A smaller area is scanned with the same number of pixels.
- The scanned pixels are smaller
- The signal is displayed on the computer screen at constant pixel size

Applications

Morphology

- The shape and size of the particles making up the object;
- Direct relation between these structures and materials properties

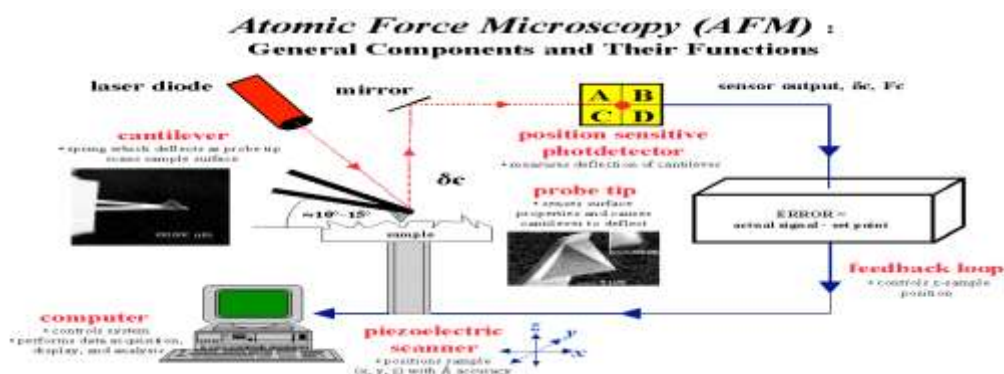
Crystallographic Information

- How the atoms are arranged in the object;
- direct relation between these arrangements and material properties

ATOMIC FORCE MICROSCOPY (AFM)

Atomic force microscopy (AFM) is a technique that is becoming widely used for both the observation and manipulation of biological macromolecules.. AFM works by moving a mechanical probe across the surface of the object being scanned. AFM therefore provides true, three-dimensional information about the object .. All are part of the more general class of techniques called scanning probe microscopy (SPM) The AFM is one of the foremost tools for imaging, measuring, and manipulating matter at the nanoscale The first Atomic Force Microscope (AFM) was developed by G. Binnig, Ch. Gerber, and C. Quate at Stanford University . A typical apparatus is depicted in Figure

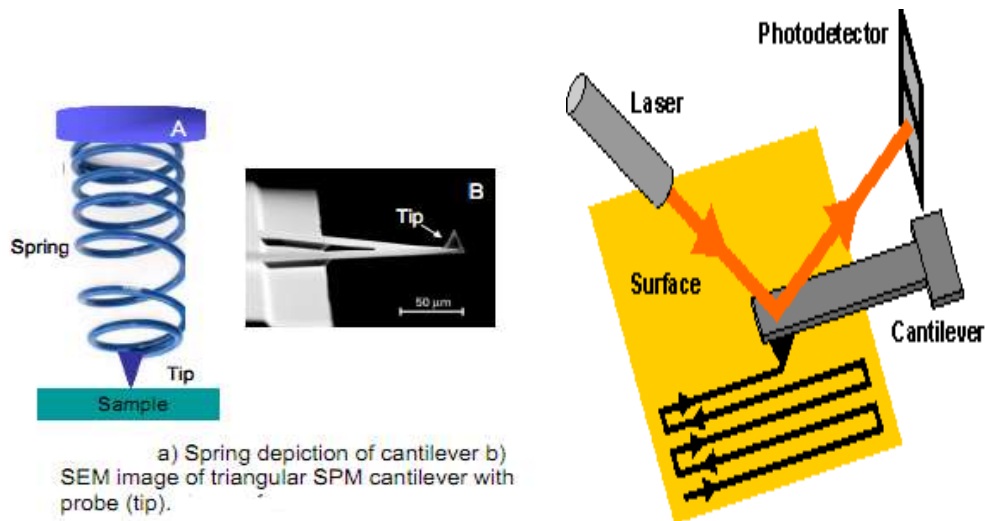
BMS COLLEGE OF ENGINEERING, BANGALORE-19
DEPARTMENT OF BIOTECHNOLOGY
Faculty : Dr. V. Saisha



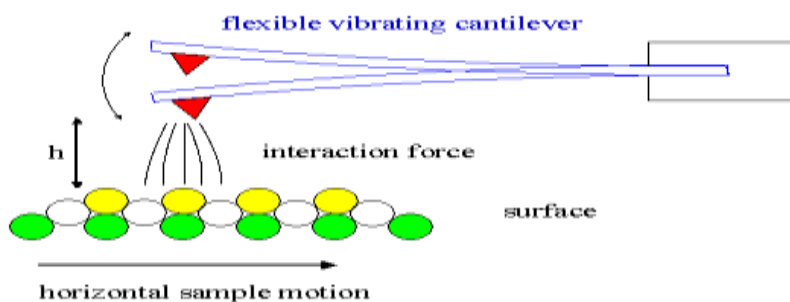
AFM provides a 3D profile of the surface on a nanoscale, by measuring forces between a sharp probe (<10 nm) and surface at very short distance. The heart of the instrument is a very fine tip (often a tungsten carbide crystal or a carbon nanotube) mounted on a springy cantilever. The AFM tip “gently” touches the surface and records the small force between the probe and the surface. The amount of force between the probe and sample is dependent on the spring constant (stiffness) of the cantilever and the distance between the probe and the sample surface. If the spring constant of cantilever (typically ~ 0.1 - 1 N/m) is less than surface, the cantilever bends and the deflection is monitored. This typically results in forces ranging from nN (10^{-9}) to μ N (10^{-6}) in the open air. The AFM cantilever can be used to measure both attractive force and repulsive force. The cantilever is designed with a very low spring constant (easy to bend) so it is very sensitive to force. At short probe-sample distances, the forces are repulsive and at large probe-sample distances, the forces are attractive. The laser is focused to reflect off the cantilever and onto the sensor. The position of the beam in the sensor measures the deflection of the cantilever and in turn the force between the tip and the sample.

In the most frequently used technique, the tapping mode, the cantilever is made to oscillate, so the tip taps its way across the sample, which has been deposited on a very smooth (usually mica) surface. The surface is scanned as it is moved in a raster fashion by a piezoelectric driver. At the same time, a laser beam is being reflected off the cantilever onto a detector mounted some distance away, giving a very long optical lever arm. As the tip taps its way across the surface and encounters a sample molecule, the difference in height is registered (see Figure). Because of the amplification of the distance by the optical lever, extremely small distances (of the order of 1 nm or less) can be measured.

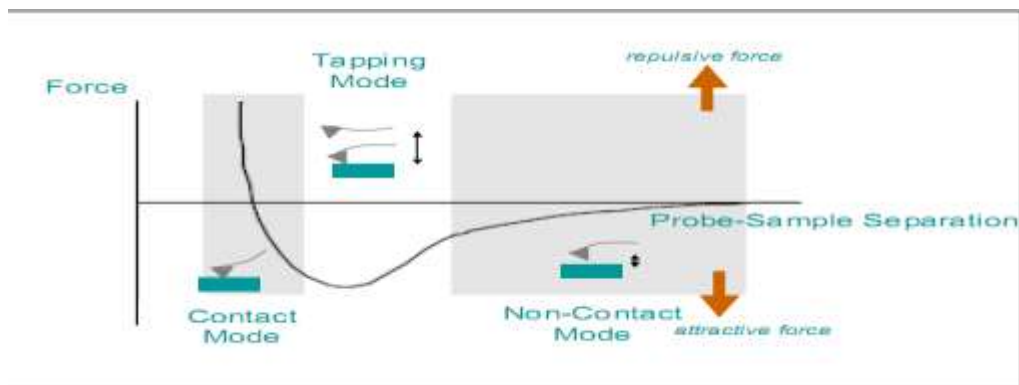
BMS COLLEGE OF ENGINEERING, BANGALORE-19
DEPARTMENT OF BIOTECHNOLOGY
Faculty : Dr. V. Saisha



AFM probes Probes are typically made from Si_3N_4 , or Si. Different cantilever lengths, materials, and shapes allow for varied spring constants. Probes may be coated with other materials for additional SPM applications such as chemical force microscopy (CFM) and magnetic force microscopy (MFM). The motion of the probe across the surface is controlled similarly to the STM using feedback loop and piezoelectric scanners. The primary difference in instrumentation design is how the forces between the probe and sample surface are monitored. The deflection of the probe is typically measured by a “beam bounce” method. A semiconductor diode laser is bounced off the back of the cantilever onto a position sensitive photodiode detector. This detector measures the bending of cantilever during the tip scanning over the sample. The measured cantilever deflections are used to generate a map of the surface topography. The dominant interactions at short probe-sample distances in the AFM are Van der Waals (VdW) interactions. During contact with the sample, the probe predominately experiences repulsive Van der Waals forces (contact mode). This leads to the tip deflection. As the tip moves further away from the surface attractive Van der Waals forces are dominant (non-contact mode).



Movement of AFM probe :contact and non -contact modes.



The molecular force is a strong function of the separation between two object The force can be monitored by the deflection of a cantilever (100-200mm long) which is in turn amplified by the deflection of a laser beam. Constant force is maintained by adjusting the z-position of the surface. A x-y scan will produce the morphology

Applications

- Digitally image a topographical surface
- Determine the roughness of a surface sample or to measure the thickness of a crystal growth layer
- Image non-conducting surfaces such as proteins and DNA
- Study the dynamic behavior of living and fixed cells

Advantages.

no fixation or staining of samples is required.

biological samples can be examined when moist, or even under aqueous conditions. For example, samples can be examined when affixed to a surface but covered by solution, allowing the effects of varying solvent compositions or the addition of substrates or other small molecules to be studied.

BMS COLLEGE OF ENGINEERING, BANGALORE-19
DEPARTMENT OF BIOTECHNOLOGY
Faculty : Dr. V. Saisha

LUMINESCENCE

Some substances emit Electromagnetic radiation at a wavelength longer than absorbed wavelength. This phenomenon is called luminescence as the light is produced at low temperature it is called as cold light. Fluorescence and Phosphorescence are two phenomena of luminescence.

Fluorescence

When a beam of light is incident on certain substances, they emit visible light / radiation at longer wavelengths. The process of emission is instantaneous and stops when incident light is cut off. These substances reemit excess radiation within 10^{-6} to 10^{-4} sec. of absorption.

In case of Phosphorescence, Light radiation is emitted continuously even after cut off of incident light. Subst. which exhibit this phenomena reemit radiation within 10^{-4} to 20 sec. longer therefore have a longer life time.

Bioluminescence is phenomenon of luminescence occurring in living system, or compound extracted from living systems. The oxidation of luminol in alkaline solution is a well known example of bioluminescence. This reaction can be used for the determination of ATP- an important molecule present in the cell.

Theory

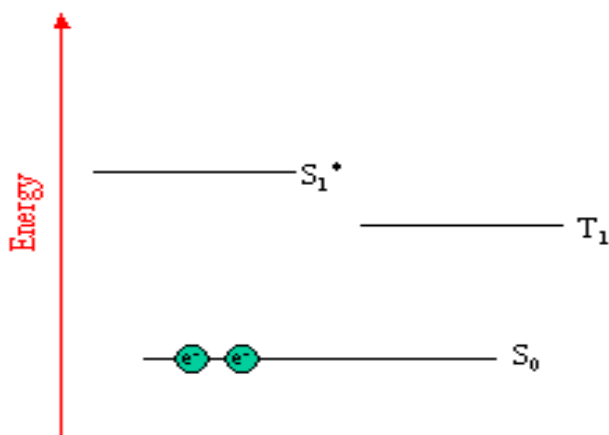
Most molecules in ground state exist in singlet state (no unpaired electrons). On absorption of radiation of suitable frequency, two conditions arise: One or more of the electrons (π electron) get raised to excited singlet state. Unpairing of electrons results in the excited triplet state.

Excited singlet electron undergoes transition to metastable triplet state and then returns to ground state. When the excited molecules come back to ground state: Three possibilities occur:

Collision deactivation - no radiation is emitted

Fluorescence (light photon) or

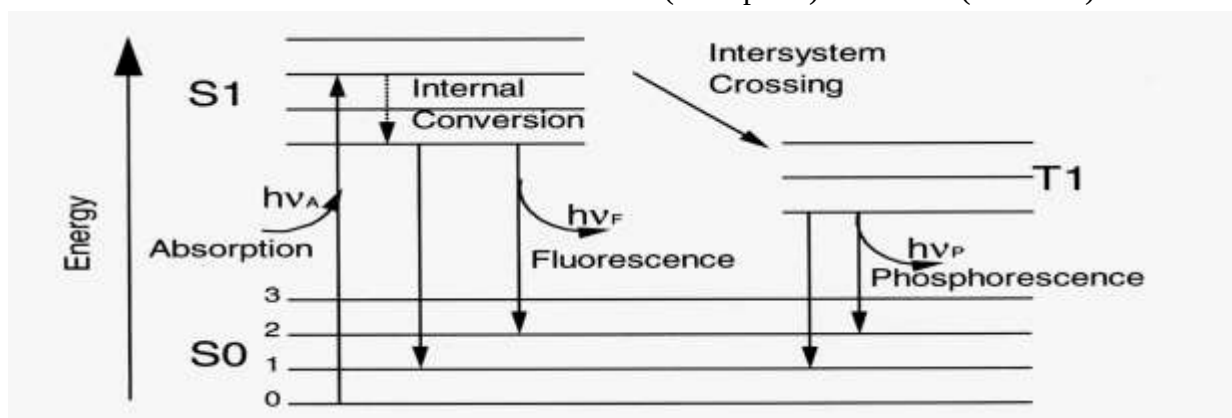
Phosphorescence



Electrons are given in the ground state electronic configuration of an atom (S_0). The first electronically excited singlet state (i.e. with electron spins anti-parallel) is S_1^* and the lower energy triplet state (i.e. with electron spins parallel) is T_1 .

BMS COLLEGE OF ENGINEERING, BANGALORE-19
DEPARTMENT OF BIOTECHNOLOGY
Faculty : Dr. V. Saisha

Lifetime of electrons in excited state is short: 10^{-13} s (absorption) and 10^{-9} s (emission).



Jablonski diagram

Factors affecting :

Molecules should have conjugate double bonds –absorb UV/visible radiation

Substituents effect the luminescence of molecules :

Electron donating groups like –NH₂., _OH enhance fluorescence

Electron withdrawing groups decrease fluorescence If a high atomic number atom is introduced it enhances phosphorescence and decreases fluorescence. pH has marked effect on fluorescence

Relationship between fluorescence intensity and concentration

$$F = K(I_0 - I)$$

K- proportionality const.

I₀- intensity of incident radiation

I –intensity of transmitted radiation

Substituting from Beer Lambert law

$$\log K I_0 / K I_0 - F = abc$$

K- fraction of incident radiation that is absorbed

a- absorptivity

b- length of cell path

c- concentration

For dilute soln. $F = 2.303 K I_0 . abc$

$$\text{Or } F = K' C$$

Relationship between phosphorescence intensity and concentration

$$\log K' I_0 / K' I_0 - P = abc$$

K- fraction of incident radiation that is absorbed

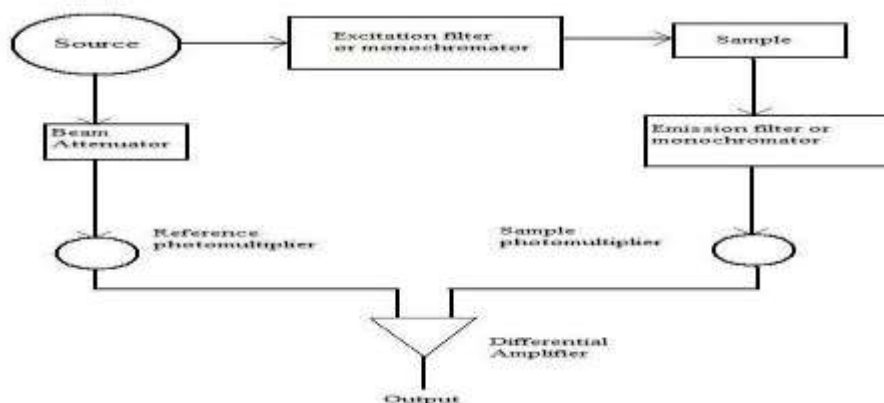
a- absorptivity

b- length of cell path

c- concentration

BMS COLLEGE OF ENGINEERING, BANGALORE-19
DEPARTMENT OF BIOTECHNOLOGY
Faculty : Dr. V. Saisha

Schematic of Fluorescence Spectrometer



Components :

Source., monochromator, detector (PMT)

Applications

Extensive applications in quantitative and qualitative analysis The quantitative applications of fluorescence measurements are due to the relationship between the fluorescence intensity and concentration of the analyte.

The fluorescence analysis methods can be broadly put under two categories as the direct and indirect methods.

In direct methods, we measure the natural fluorescence of the analyte In the indirect methods, we either suitably derivatise the analyte to be determined to a fluorescent species or explore for a fluorescent species whose fluorescence is quenched by the analyte. The vast range of applications in the area of inorganic species can be put into three groups. The first group includes the inorganic species that have an intrinsic fluorescence; the other two groups are of the species that give fluorescence on reacting with inorganic and organic reagents, respectively. Several minerals like calcite, fluorite, rubies and zircon on exposure to UV radiation start emitting fluorescence in the absence of fluorescence quenchers.

Egs.

detection of metal ions in environmental and biological samples.

determination of NO-NO₂ and SO₂ as atmospheric pollutants.

One can detect as small as 1 ppb of NO₂ or 5 ppb of SO₂ gas by these methods.

the presence of trace amounts of metal pollutants in water samples

The applications of phosphorescence are limited due to the lack of suitable instrumentation and the requirement of making measurements at cryogenic temperatures. However, with the

BMS COLLEGE OF ENGINEERING, BANGALORE-19
DEPARTMENT OF BIOTECHNOLOGY
Faculty : Dr. V. Saisha

introduction of new instrumental methods and the advances made in room temperature phosphorescence, phosphorimetry is making inroads in the domain of clinical chemistry and in the areas of forensic, environmental and pharmaceutical sciences.

CALORIMETRY

Is the science of measuring the heat of chemical reactions or physical changes. The word calorimetry is derived from the Latin word calor, meaning heat.

Scottish physician and scientist Joseph Black, is said to be the founder of calorimetry. Calorimetric measurements of biochemical reactions such as protein–DNA binding, protein–protein complex formation and protein unfolding is called biocalorimetry.

Principle

When two bodies at different temperatures are mixed, heat will be transferred from body at higher temperature to body at lower temperature. The body at higher temperature releases heat while body at lower temperature absorbs it, so that: Heat lost = Heat gained, It happens till both acquire same temperature.

In a system, the possibilities of energy transfer are:

(a) Temperature of mixture (T) is always > lower temperature (TL) and < higher temperature (TH), i.e., $TL < T < TH$ i.e. the temperature of mixture can never be lesser than lower temperatures and greater than higher temperature.

(b) When temperature of a body changes, the body releases heat if its temperature falls and absorbs heat when its temperature rises. The heat released or absorbed by a body of mass m is given by: $Q = mc\Delta T$, Where c is specific heat of the body and ΔT change in its temperature in °C or K.

(c) When state of a body changes, change of state takes place at constant temperature and heat released or absorbed is given by $Q = mL$, Where L is latent heat. Heat is absorbed if solid converts into liquid or liquid converts into vapours and is released if liquid converts into solid or vapours converts into liquid.

The heat capacity (also known as the specific heat) of a material is a measure of its ability to absorb heat. It is defined as C_p (the subscript denotes constant pressure) the quantity of heat which will change the temperature of exactly 1 g of a substance by 1 °C. For most reactions involving low molecular mass reagents, the values for C_p of reactants and products are not significantly different. However, for large molecules such as proteins, there may be significant changes in C_p .

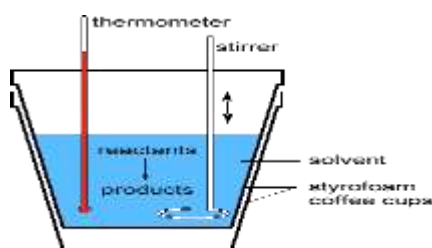
Calorimetry experiments determines the heat/ enthalpy changes of reactions by making accurate measurements of temperature changes in a calorimeter. A calorimeter is an insulated device used for measuring the amount of heat absorbed or released in a chemical reaction or physical process.

Two types: coffee cup calorimeter., bomb calorimeter

Heat flow is calculated using the relation : $q = (\text{specific heat}) \times m \times \Delta t$ where q is heat flow, m is mass in grams, and Δt is the change in temperature.

Coffee cup calorimeter

BMS COLLEGE OF ENGINEERING, BANGALORE-19
DEPARTMENT OF BIOTECHNOLOGY
Faculty : Dr. V. Saisha



A coffee cup calorimeter is essentially a polystyrene (Styrofoam) cup with a lid. The cup is partially filled with a known volume of water and a thermometer is inserted through the lid of the cup so that its bulb is below the water surface. When a chemical reaction occurs in the coffee cup calorimeter, the heat of the reaction is calculated. Since the reaction takes place in water, water acts as the surrounding. Heat changes in the water are called q_{surr} while heat changes in the reaction are called q_{rxn} . The temperature of the water is taken before the reaction takes place, and it is recorded as the initial temperature T_i . The final temperature of water is recorded at T_f . The change in the water temperature is used to calculate the amount of heat that has been absorbed or evolved in the reaction. The amount of heat absorbed/ released in water is determined using the mass of water m , specific heat of water C , and change in water temperature. $q_{\text{surr}} = m \times C \times (T_f - T_i)$

The insulation provided by the styrofoam ensures that any heat absorbed or released by the system (reactants and products) goes only to the solvent in the cup. The thermometer allows the ΔT of the surroundings to be measured. By the First Law of Thermodynamics $-q_{\text{system}} = q_{\text{surroundings}}$

Using ΔT , which can be measured, and the heat capacity of the solvent in the coffee cup, the heat lost by the system can be calculated.

Merits:

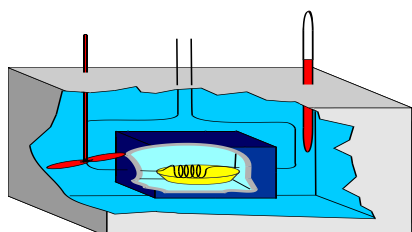
Efficient in measuring heat flow in a solution

Demerits:

can't be used for reactions which involve gases

can't be used for high temperature reactions

Bomb Calorimeter



A bomb calorimeter works in the same manner with only a difference i.e. the reaction takes place in a sealed metal container, which is placed in the water in an insulated container. Heat flow from the reaction crosses the walls of the sealed container to the water. The temperature difference of the water is measured, just as it was for a coffee cup calorimeter. Analysis of the heat flow is a bit more complex as heat flow into the metal parts of the calorimeter must be taken into account.

BMS COLLEGE OF ENGINEERING, BANGALORE-19
DEPARTMENT OF BIOTECHNOLOGY
Faculty : Dr. V. Saisha

$Q_{\text{reaction}} = - (q_{\text{water}} + q_{\text{bomb}})$, where $q_{\text{water}} = 4.18 \text{ J/(g} \cdot ^\circ\text{C)} \times m_{\text{water}} \times \Delta t$

The bomb has a fixed mass and specific heat. The mass of the bomb multiplied by its specific heat is sometimes termed the **calorimeter constant**, denoted by the symbol C with units of joules per degree Celsius. The calorimeter constant is determined experimentally ($q_{\text{bomb}} = C \times \Delta t$)

DIFFERENTIAL SCANNING CALORIMETRY

Differential scanning calorimetry or **DSC** is a thermoanalytical technique developed by E.S. Watson and M.J. O'Neill in 1962. DSC involves measurement of changes in C_p as a function of change in temperature. It is especially useful in determining thermodynamic parameters of phenomena initiated by an increase or a decrease in temperature. The effects of different experimental conditions such as pH, ionic strength, solvent identity, presence/absence of ligands on these parameters can be studied by DSC. The technique is especially sensitive to phase changes in biopolymers arising during phenomena such as protein-protein binding, protein unfolding and ligand binding. Because very small ΔC_p values are usually measured, it is necessary to compare a solution of the sample to a reference solution containing only buffer. Moreover, repeated scans need to be made over a period of time to build up sufficient data for accurate C_p determination. Both the sample and reference are maintained at nearly the same temperature throughout the experiment. When the sample undergoes a physical transformation such as phase transitions, more or less heat will need to flow to it than the reference to maintain both at the same temperature. Whether less or more heat must flow to the sample depends on whether the process is exothermic or endothermic. For example, as a solid sample melts to a liquid it will require more heat flowing to the sample to increase its temperature at the same rate as the reference. This is due to the absorption of heat by the sample as it undergoes the endothermic phase transition from solid to liquid. Likewise, as the sample undergoes exothermic processes (such as crystallization) less heat is required to raise the sample temperature. By observing the difference in heat flow between the sample and reference, differential scanning calorimeters are able to measure the amount of heat absorbed or released during such transitions. DSC may also be used to observe more subtle physical changes, such as glass transitions.

Applications

DSC has been used in the study of a wide range of biochemical phenomena. In general, any process involving a temperature-induced change of phase in a biomacromolecule is amenable to study by this technique. These would include double-to single-strand transitions in DNA, unfolding of globular proteins and processes involving multi-domain/multi-subunit proteins. It is possible to vary the experimental conditions used such as pH, ion strength or by including stabilizing molecules. DSC can also be used to resolve effects involving different protein domains and hence as a tool to detect interdomain cooperativity. The technique is not limited to proteins and has been widely used in studies of polysaccharides and even nonbiological polymers.

BMS COLLEGE OF ENGINEERING, BANGALORE-19
DEPARTMENT OF BIOTECHNOLOGY
Faculty : Dr. V. Saisha

DSC measures the temperatures and heat flow associated with transitions in materials as a function of time and temperature in a controlled atmosphere. These measurements provide quantitative and qualitative information about the physical and chemical changes that involve endothermic or exothermic processes or changes in heat capacity.

Biological applications

DSC directly measures heat changes that occur in biomolecules during controlled increase or decrease in temperature, making it possible to study materials in their native state. DSC measures the enthalpy (ΔH) of unfolding due to heat denaturation. A biomolecule in solution is in equilibrium between the native (folded) conformation and its denatured (unfolded) state. The higher the thermal transition midpoint (T_m), when 50% of the biomolecules are unfolded, the more stable the molecule.

DSC is also used to determine the change in heat capacity (ΔC_p) of denaturation directly measures the stability and unfolding of a protein, lipid, or nucleic acid. Transition midpoint - T_m Enthalpy (ΔH) and heat capacity change (ΔC_p) associated with unfolding.

DSC can elucidate the factors that contribute to the folding and stability of native biomolecules, including hydrophobic interactions, hydrogen bonding, conformational entropy, and the physical environment.

A biomolecule in aqueous solution is in equilibrium between the native (folded) conformation and its denatured (unfolded) conformation. The stability of the native state is based on the magnitude of the Gibbs free energy (ΔG) of the system and the thermodynamic relationships between enthalpy (ΔH) and entropy (ΔS) changes. A positive ΔG indicates the native state is more stable than the denatured state – the more positive the ΔG , the greater the stability. For a protein to unfold, stabilizing forces need to be broken. Conformational entropy overcomes stabilizing forces allowing the protein to unfold at temperatures where entropy becomes dominant.

DSC measures ΔH of unfolding due to heat denaturation. The transition midpoint T_m is the temperature where 50% of the protein is in its native conformation and the other 50% is denatured. The higher the T_m , the more stable the molecule. During the same experiment DSC also measures the change in heat capacity (ΔC_p) for denaturation. Heat capacity changes associated with protein unfolding are primarily due to changes in hydration of side chains that were buried in the native state, but become solvent exposed in the denatured state. Many factors are responsible for the folding and stability of native biopolymers, including hydrophobic interactions, hydrogen bonding, conformational entropy, and the physical environment (pH, buffer, ionic strength, excipients, etc.)

Other Applications

Liquid biopharmaceutical formulations.

Process development.

Protein engineering.

Rank order binding.

Antibody domain studies.

Characterization of membranes, lipids, nucleic acids and micellar systems.

BMS COLLEGE OF ENGINEERING, BANGALORE-19
DEPARTMENT OF BIOTECHNOLOGY
Faculty : Dr. V. Saisha

Assessment of the effects of structural change on a molecule's stability.

Measurement of ultra-tight molecular interactions (up to 10^{20} M^{-1}).

Assessment of biocomparability during manufacturing.

Heats of fusion and reactions

Specific heat capacity

Oxidative/thermal stability

Reaction kinetics

Purity

BMS COLLEGE OF ENGINEERING, BANGALORE-19
DEPARTMENT OF BIOTECHNOLOGY
Faculty : Dr. V. Saisha

MASS SPECTROMETRY

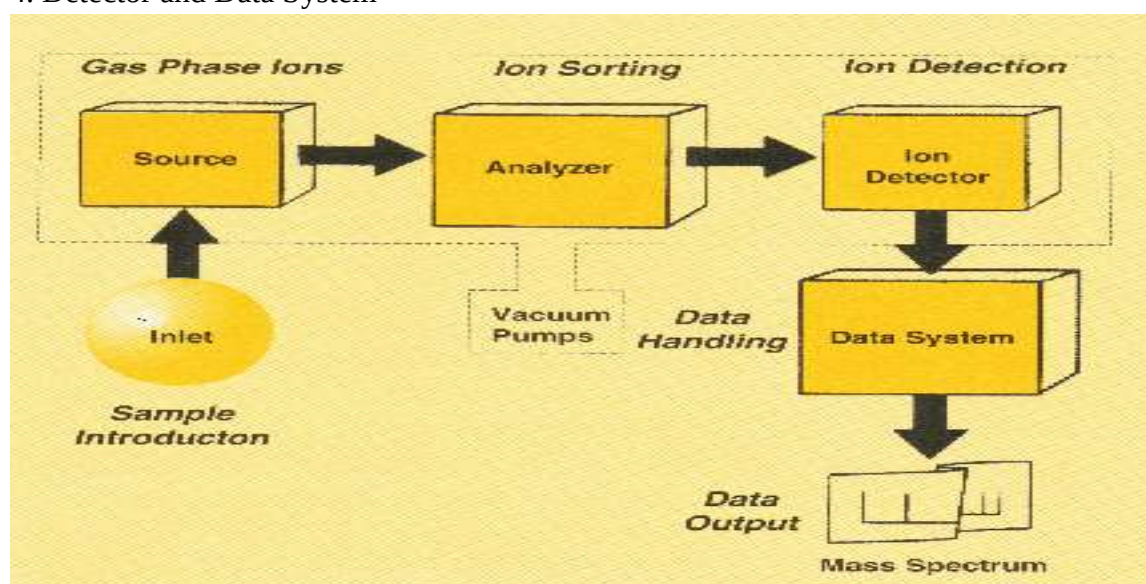
Mass spectrometry is a powerful technique for chemical analysis that is used to identify unknown compounds, to quantify known compounds, and to elucidate molecular structure. It measures molecular and atomic masses of whole molecules, molecular fragments and atoms by generation and detection of the corresponding gas phase ions, separated according to their mass-to-charge ratio (m/z). Mass spectrometry is based upon the motion of a charged particle, called an ion, in an electric or magnetic field. The mass to charge ratio (m/z) of the ion effects this motion. Since the charge of an electron is known, the mass to charge ratio a measurement of an ion's mass. It is dependent on the thermodynamic stability of ions produced and collected. Limitation – is a “Destructive” technique – cannot reclaim sample

History

- JJ Thomson built MS prototype to measure m/z of electron, awarded Nobel Prize in 1906
- MS concept first put into practice by Francis Aston, a physicist working in Cambridge England in 1919
- Designed to measure mass of elements
- Aston Awarded Nobel Prize in 1922 1948-52 - Time of Flight (TOF) mass analyzers introduced
- 1955 - Quadrupole ion filters introduced by W. Paul, also invents the ion trap in 1983 (wins 1989 Nobel Prize)
- 1968 - Tandem mass spectrometer appears

Components of a MS

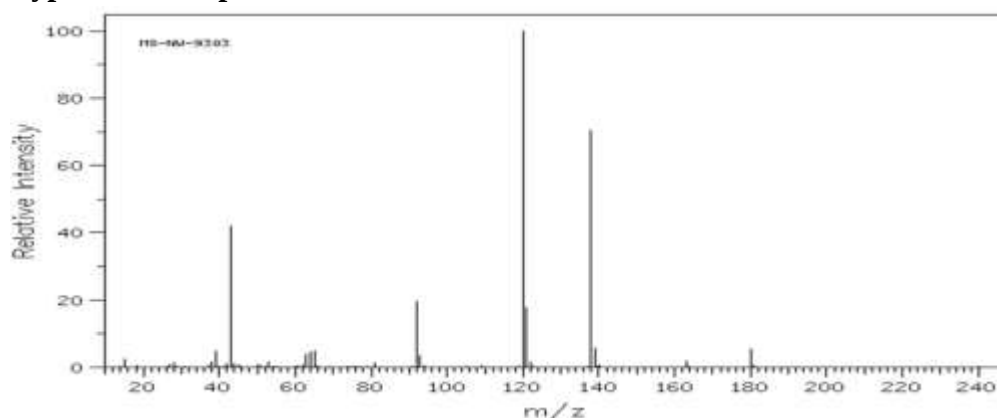
1. Sample Inlet
2. Ion Source
3. Mass Analyzer
4. Detector and Data System



BMS COLLEGE OF ENGINEERING, BANGALORE-19
DEPARTMENT OF BIOTECHNOLOGY
Faculty : Dr. V. Saisha

All mass spectrometers have a vacuum system to maintain the low pressure, which is also called high vacuum, required for operation. High vacuum minimizes ion-molecule reactions, scattering, and neutralization of the ions.

Typical Mass Spectrum



Characterized by sharp, narrow peaks. X-axis position indicates the m/z ratio of a given ion (for singly charged ions this corresponds to the mass of the ion) Height of peak indicates the relative abundance of a given ion (not reliable for quantitation)

Peak intensity indicates the ion's ability to desorb or "fly" (some fly better than others)

Resolution & Resolving Power

- Width of peak indicates the resolution of the MS instrument
- The better the resolution or resolving power, the better the instrument and the better the mass accuracy
- Resolving power is defined as: $\frac{M}{\Delta M}$ M is the mass number of the observed mass (ΔM) is the difference between two masses that can be separated

Sample introduction:

Samples are introduced either as a gas to be ionized in the ion source, or by ejection of charged molecular species from a solid surface or solution. sample introduction and ionization can occur simultaneously.

The selection of a sample inlet depends upon the sample and the sample matrix. Most ionization techniques are designed for gas phase molecules so the inlet must transfer the analyte into the source as a gas phase molecule. If the analyte is sufficiently volatile and thermally stable, a variety of inlets are available. Gases and samples with high vapor pressure are introduced directly into the source region. Liquids and solids are usually heated to increase the vapor pressure for analysis. If the analyte is thermally labile (it decomposes at high temperatures) or if it does not have a sufficient vapor pressure, the sample must be directly ionized from the condensed phase. These direct ionization techniques require special instrumentation and are more difficult to use. However, they greatly extend the range of compounds that may be analyzed by mass spectrometry. Commercial instruments are available that use direct ionization techniques to routinely analyze proteins and polymers with molecular weights greater than 100,000 dalton. Vapors of sample are flushed from the solid or liquid matrix with a stream of carrier gas and trapped on an adsorbing column. The trapped vapors are subsequently

BMS COLLEGE OF ENGINEERING, BANGALORE-19
DEPARTMENT OF BIOTECHNOLOGY
Faculty : Dr. V. Saisha

desorbed by programmed heating of the trap and introduced into the mass spectrometer by a capillary connection. Gas and liquid chromatographs are widely used as sample inlet devices for mass spectrometers.

Direct Vapor Inlet. The simplest sample introduction method is a direct vapor inlet. The gas phase analyte is introduced directly into the source region of the mass spectrometer through a needle valve. Pump out lines are usually included to remove air from the sample. This inlet works well for gases, liquids, or solids with a high vapor pressure. Samples with low vapor pressure are heated to increase the vapor pressure. Since this inlet is limited to stable compounds and modest temperatures, it only works for some samples.

Gas Chromatography. Gas chromatography is probably the most common technique for introducing samples into a mass spectrometer. Complex mixtures are routinely separated by gas

chromatography and mass spectrometry is used to identify and quantitate the individual components. Several different interface designs are used to connect these two instruments. The most significant characteristics of the inlets are the amount of GC carrier gas that enters the mass spectrometer and the amount of analyte that enters the mass spectrometer. If a large flow of GC carrier gas enters the mass spectrometer it will increase the pressure in the source region. Maintaining the required source pressure will require larger and more expensive vacuum pumps.

The amount of analyte that enters the mass spectrometer is important for improving the detection

limits of the instrument. Ideally all the analyte and none of the GC carrier gas would enter the source region.

The most common GC/MS interface now uses a capillary GC column. Since the carrier gas flow rate is very small for these columns, the end of the capillary is inserted directly into the

source region of the mass spectrometer. The entire flow from the GC enters the mass spectrometer. Since capillary columns are now very common, this inlet is widely used.

However, wide bore capillaries and packed GC columns have higher flow rates. This significantly increases the pressure in the mass spectrometer. Several inlet designs are available to reduce the gas flow into the source. The simplest design splits the GC effluent so that only a

small portion of the total flow enters the mass spectrometer. Although this inlet reduces the gas load on the vacuum system, it also reduces the amount of analyte. Effusive separators and membrane inlets are more selective and transport a higher fraction of the analyte into the source region. Each of these methods has efficiency and resolution drawbacks but they are necessary for some experiments.

Liquid Chromatography. Liquid chromatography inlets are used to introduce thermally labile compounds not easily separated by gas chromatography. These inlets have undergone considerable development and are now fairly routine. Because these inlets are used for temperature sensitive compounds, the sample is ionized directly from the condensed phase.

BMS COLLEGE OF ENGINEERING, BANGALORE-19
DEPARTMENT OF BIOTECHNOLOGY
Faculty : Dr. V. Saisha

Direct Insertion Probe. The Direct Insertion Probe (DIP) is widely used to introduce low vapor pressure liquids and solids into the mass spectrometer. The sample is loaded into a short capillary tube at the end of a heated sleeve. This sleeve is then inserted through a vacuum lock so the sample is inside the source region. After the probe is positioned, the temperature of the capillary tube is increased to vaporize the sample. This probe is used at higher temperatures than

are possible with a direct vapor inlet. In addition, the sample is under vacuum and located close to the source so that lower temperatures are required for analysis. This is important for analyzing

temperature sensitive compounds. Although the direct insertion probe is more cumbersome than

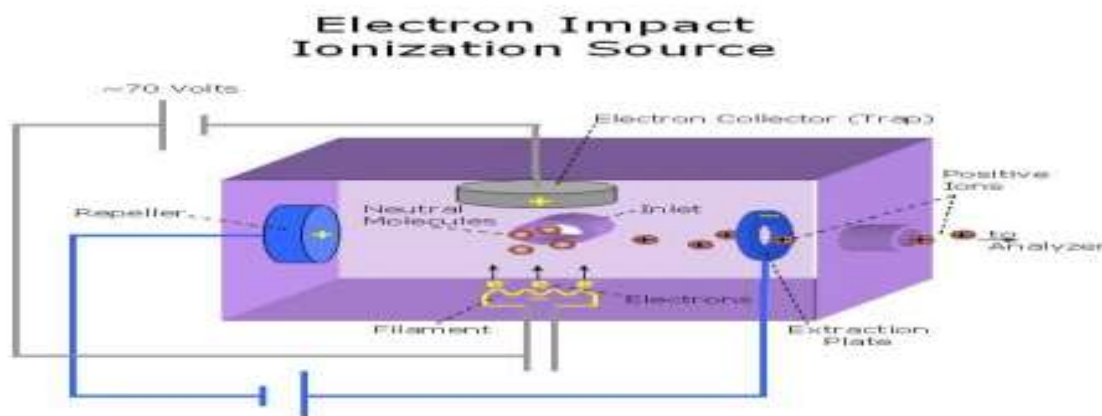
the direct vapor inlet, it is useful for a wider range of samples.

Ionization techniques:

1. Electron impact ionization
2. Chemical ionization
3. Field ionization
4. Ion desorption methods
5. Ion evaporation methods

Most ionization techniques excite the neutral analyte molecule which then ejects an electron to form a radical cation ($M^+ \cdot$). Other ionization techniques involve ion molecule reactions that produce adductions (MH^+). The most important considerations are the physical state of the analyte and the ionization energy. Electron ionization and chemical ionization are only suitable for gas phase ionization. Fast atom bombardment, secondary ion mass spectrometry, electrospray, and matrix assisted laser desorption are used to ionize condensed phase samples... Some ionization techniques are very soft and only produce molecular ions,* other techniques are very energetic and cause ions to undergo extensive fragmentation. Although this fragmentation complicates the mass spectrum, it provides structural information for the identification of unknown compounds.

Electron impact Ionization



Electron impact Ionization (EI) is the most common ionization technique used for mass

BMS COLLEGE OF ENGINEERING, BANGALORE-19
DEPARTMENT OF BIOTECHNOLOGY
Faculty : Dr. V. Saisha

spectrometry. EI works well for many gas phase molecules, but it does have some limitations. Although the mass spectra are very reproducible and are widely used for spectral libraries, EI causes extensive fragmentation so that the molecular ion is not observed for

many compounds. Fragmentation is useful because it provides structural information for interpreting unknown spectra. The electrons used for ionization are produced by passing a current through a wire filament. The amount of current controls the number of electrons emitted by the filament. An electric field accelerates these electrons across the source region to produce a beam of high energy electrons. When an analyte molecule passes through this electron beam, a valence shell electron can be removed from the molecule to produce an ion. Ionization does not occur by electron capture, which is highly dependent upon molecular structure. Instead, EI produces positive ions by knocking a valence electron off the analyte molecule. As the electron passes close to the molecule the negative charge of the electron repels and distorts the electron cloud surrounding the molecule. This distortion transfers kinetic energy from the fast-moving electron to the electron cloud of the molecule. If enough energy is transferred by the process, the molecule will eject a valence electron and form a radical cation ($M^+_{\cdot}$).

Since the ionization is produced by a single electron that is accelerated to 70 V, this is commonly referred to as 70 eV EI. This is enough energy to cause extensive fragmentation, and

at this level small changes in the electron energy do not significantly effect the fragmentation patterns. The distribution of energy transferred during ionization and the large number of fragmentation pathways results in a variety of products for a given analyte. Other electron voltages may be used to vary the amount of fragmentation produced during ionization. For most organic compounds the threshold energy for EI is about 10 eV. Because a mass spectrum is produced by ionizing many molecules, the spectrum is a distribution of the possible product ions. Intact molecular ions are observed from ions produced with little excess energy. Other molecular ions have more energy and undergo fragmentation in the source region. The abundance of the resulting fragments, often called product ions, is determined by the kinetics of the fragmentation pathways and the ionization energy. Changing the ionization energy changes the observed distribution of fragment ions. This distribution provides the structural information for interpreting mass spectra

Chemical Ionization.

Chemical Ionization (CI) is a "soft" ionization technique that produces ions with little excess energy. As a result, less fragmentation is observed in the mass spectrum. Since this increases the abundance of the molecular ion, the technique is complimentary to 70 eV EI. CI is often used to verify the molecular mass of an unknown. Only slight modifications of an EI source region are required for CI experiments.

In Chemical Ionization the source is enclosed in a small cell with openings for the electron beam, the reagent gas and the sample. The reagent gas is added to this cell at approximately 10 Pa (0.1 torr) pressure. This is higher than the 10^{-3} Pa (10^{-5} torr) pressure typical for a mass spectrometer source. At 10^{-3} Pa the mean free path between collisions is approximately 2 meters and ion-molecule reactions are unlikely. In the CI source, however, the mean free path

between collisions is only 10⁻⁴ meters and analyte molecules undergo many collisions with the reagent gas. The reagent gas in the CI source is ionized with an electron beam to produce a cloud of ions. The reagent gas ions in this cloud react and produce adduct ions like CH₃⁺ which are excellent proton donors. When analyte molecules (M) are introduced to a source region with this cloud of ions, the reagent gas ions donate a proton to the analyte molecule and produce MH⁺ ions. The energetics of the proton transfer is controlled by using different reagent gases. The most common reagent gases are methane, isobutane and ammonia. Methane is the strongest proton donor commonly used with a proton affinity (PA) of 5.7 eV. For softer ionization, isobutane (PA 8.5 eV) and ammonia (PA 9.0 eV) are frequently used. Acid base chemistry is frequently used to describe the chemical ionization reactions. The reagent gas must be a strong enough Brønsted acid to transfer a proton to the analyte. Fragmentation is minimized in CI by reducing the amount of excess energy produced by the reaction. Because the adduct ions have little excess energy and are relatively stable, CI is very useful for molecular mass determination. Some typical reactions in a CI source are shown in Figure 5.

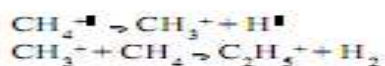
A) EI of reagent gas to form ions:



B) Reaction of reagent gas ions to form adducts:



OR



C) Reaction of Reagent Gas Ions with analyte molecules:

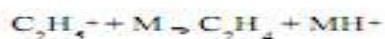
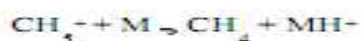


Figure 5 Chemical Ionization Reactions

Field Ionization the Sample is introduced in vapour state and is subjected to intense electric fields. In intense E field (10⁷-10⁹ V/cm) outer bonding e are subject to large forces sufficient to overcome ionization potential Electron is removed to generate a molecular radical cation

Ion desorption methods

Introduction of solid samples which are volatile Subst should be volatile , volatilize prior to ionization Common method is DIP (direct insertion probe)

Different methods :

Field desorption : Tungsten wire coated with organic material from which carbon filaments or dendrites are grown .The sample is coated onto dendrites to form solid coat Intense Electric field under low pressure is applied .under these conditions Ions are desorbed and accelerated into analyzer.

BMS COLLEGE OF ENGINEERING, BANGALORE-19
DEPARTMENT OF BIOTECHNOLOGY
Faculty : Dr. V. Saisha

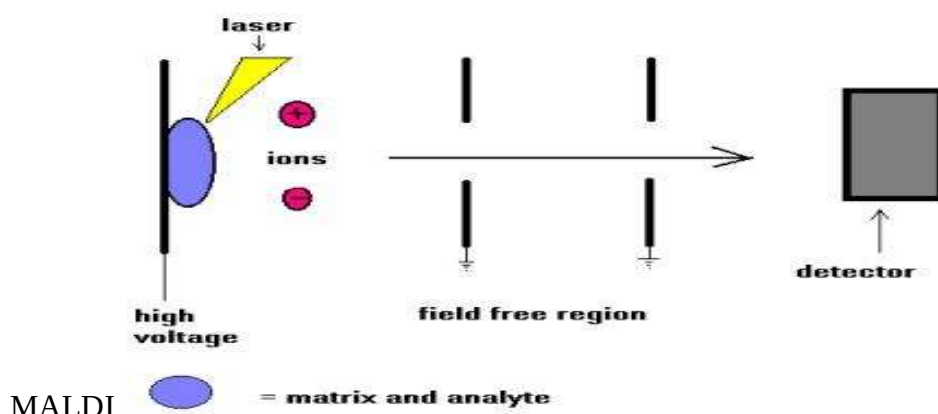
Fast atom bombardment ionization : Fast Atom Bombardment. use high energy atoms to sputter and ionize the sample in a single step., a beam of rare gas neutrals (FAB) is focused on the liquid or solid sample. The impact of this high energy beam causes the analyte molecules to sputter into the gas phase and ionize in a single step. The exact mechanism of this process is not well understood, but these techniques work well for compounds with molecular weights up to a few thousand dalton. Since no heating is required, sputtering techniques (especially FAB) are useful for studying thermally labile compounds that decompose in conventional inlets .. InFAB the analyte is dissolved in a liquid matrix. A drop of the sample/matrix mixture is placed at the end of an insertion probe and introduced to the source region. The fast atom beam is focused on this droplet to produce analyte ions. Glycerol or similar low vapor pressure liquids are typically used for the matrix. Ideally, the analyte is soluble in the liquid matrix and a monolayer of analyte forms on the surface of the droplet. According to one theory, this monolayer concentrates the analyte while the dissolved sample provides a reservoir to replenish the monolayer as the analyte is depleted. Without this constant replenishment from the bulk solution, the ionizing beam will rapidly deplete the analyte and the signal is difficult to observe. **Plasma desorption** is a Pulsed technique. The Samples are coated onto suitable surface (Mylar,nafion)Bombardment with plasma particles results in ionization . TOF of ion is measured .

Laser desorption

Matrix Assisted Laser Desorption/Ionization (MALDI) is used to analyze extremely large molecules. This technique directly ionizes and vaporizes the analyte from the condensed phase. MALDI is often used for the analysis of synthetic and natural polymers, proteins, and peptides. Analysis of compounds with molecular weights up to 200,000 dalton is possible and this high mass limit is continually increasing.

In MALDI, both desorption and ionization are induced by a single laser pulse The sample is prepared by mixing the analyte and a matrix compound chosen to absorb the laser wavelength. This is placed on a probe tip and dried. A vacuum lock is used to insert the probe into the source region of the mass spectrometer. A laser beam is then focused on this dried mixture and the energy from a laser pulse is absorbed by the matrix. This energy ejects analyte ions from the surface so that a mass spectrum is acquired for each laser pulse.

Typical MALDI spectra include the molecular ion,some multiply charged ions, and very few fragments.



MALDI

Ion evaporation methods

Spray of charged liquid droplets produced by nebulization or atomization. Sample is solvated by the charged drop. On evaporation in high vacuum, drop size decreases and charge resides on surface of sample.

Types :

- Thermospray ionization sources
- Electrospray and ion spray ionization
- Ionization at atmp. Pressure (API)

Atmospheric Pressure Ionization.

Atmospheric Pressure Ionization (API) sources ionize the sample at atmospheric pressure and then transfer the ions into the mass spectrometer. These techniques are used to ionize thermally labile samples such as peptides, proteins and polymers directly from the condensed phase. The sample is dissolved in an appropriate solvent and this solution is introduced into the mass spectrometer. With conventional inlets the solvent increases the pressure in the source region of the mass spectrometer. API sources introduce the sample through a series of differentially pumped stages. This maintains the large pressure difference between the ion source and the mass spectrometer without using extremely large vacuum pumps. In addition a drying gas is used to break up the clusters that form as the solvent evaporates. Because the analyte molecules have more momentum than the solvent and air molecules, they travel through the pumping stages to the mass analyzer.

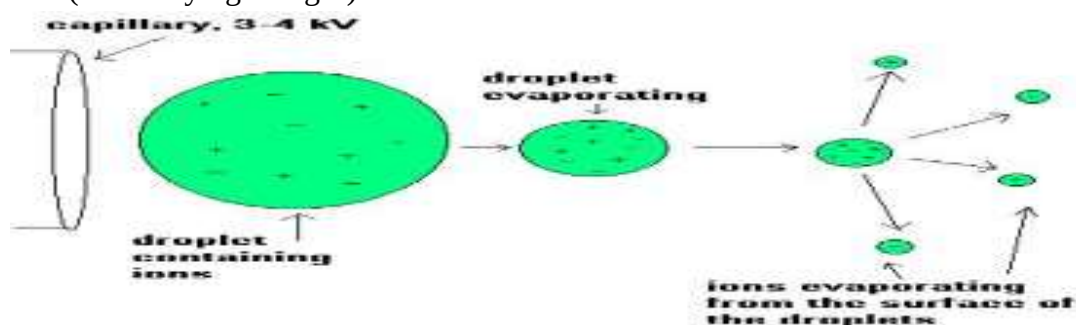
. Electrospray Ionization

Sample is dissolved in polar, volatile buffer (no salts) and pumped through a stainless steel capillary (70 - 150 mm) at a rate of 10-100 mL/min. Strong voltage (3-4 kV) applied at tip along with flow of nebulizing gas causes the sample to “nebulize” or aerosolize. As the liquid leaves the nozzle, the electric field induces a net charge on the small droplets. As the solvent evaporates, the droplet shrinks and the charge density at the surface of the droplet increases. The droplet finally reaches a point where the coulombic repulsion from this electric charge is greater than the surface tension holding it together. This causes the droplet to explode and produce multiply charged analyte ions. Because electrospray produces multiply charged ions, high molecular weight compounds are observed at lower m/z value. This increases the mass

BMS COLLEGE OF ENGINEERING, BANGALORE-19
DEPARTMENT OF BIOTECHNOLOGY
Faculty : Dr. V. Saisha

range of the analyzer so that higher molecular weight compounds may be analyzed with a less expensive mass spectrometer.

Aerosol is directed through regions of higher vacuum until droplets evaporate to near atomic size (still carrying charges)



Can be modified to “nanospray” system with flow < 1 mL/min Very sensitive technique, requires less than a picomole of material. Strongly affected by salts & detergents. Positive ion mode measures $(M + H)^+$ (add formic acid to solvent). Negative ion mode measures $(M - H)^-$ (add ammonia to solvent) If the sample has functional groups that readily accept H^+ (such as amide and amino groups found in peptides and proteins) then positive ion detection is used-eg PROTEINS If a sample has functional groups that readily lose a proton (such as carboxylic acids and hydroxyls as found in nucleic acids and sugars) then negative ion detection is used-eg DNA

Ionization Techniques

Short name	Full name	How it works
EI	Electron Ionization	Impact of 70 eV electrons
CI	Chemical Ionization	Transfer of H^+ from CH_5^+
FI	Field Ionization	High Field (12kV)
FAB	Fast Atom Bombardment	Beam of Xenon atoms
FD	Field Desorption	High Field (12kV)
MALDI	Matrix-Assisted Laser Desorption Ionization	Laser hits sample in UV-absorbing matrix
ESI	Electrospray Ionization	Spray and desolvation of preformed ions

Soft Ionization Methods Both MALDI and ESI are considered as Soft ionization techniques as they keep the molecule of interest fully intact.

Made it possible to analyze large molecules via inexpensive mass analyzers such as quadrupole, ion trap and TOF

Electro-spray ionization first conceived in 1960's by Malcolm Dole but put into practice in 1980's by John Fenn (Yale)

MALDI first introduced in 1985 by Franz Hillenkamp and Michael Karas (Frankfurt)

In Matrix-assisted laser desorption ionization (MALDI), the Analyte (protein) is mixed with large excess of matrix (small organic molecule),-Irradiated with short pulse of laser light.

BMS COLLEGE OF ENGINEERING, BANGALORE-19
DEPARTMENT OF BIOTECHNOLOGY
Faculty : Dr. V. Saisha

Wavelength of laser is the same as absorbance max of matrix. In electrospray mass spectrometry (ESI-MS)-Liquid containing analyte is forced through a steel capillary at high voltage to electrostatically disperse analyte. Charge imparted from rapidly evaporating liquid. Unlike ESI, MALDI generates spectra that have just a singly charged ion: Positive mode generates ions of $M + H$, Negative mode generates ions of $M - H$. It is more robust than ESI (tolerates salts and nonvolatile components). Easier to use and maintain, capable of higher throughput and Requires 10 mL of 1 pmol/mL sample (small vol.)

Mass analyzers:

After ions are formed in the source region they are accelerated into the mass analyzer by an electric field. The mass analyzer separates these ions according to their m/z value. The selection of a mass analyzer depends upon the resolution mass range, scan rate and detection limits required for an application. Each analyzer has very different operating characteristics.

Types of Analysers

- Magnetic sector analyzer
- Electric sector analyser
- Quadrupole mass filters
- Ion traps
- **Time-of-Flight Analyzer (TOF)**

Analyzers are typically described as either continuous or pulsed. Continuous analyzers include quadrupole filters and magnetic sectors. They transmit a single selected m/z to the detector and the mass spectrum is obtained by scanning the analyzer so that different mass to charge ratio ions are detected. While a certain m/z is selected, any ions at other m/z ratios are lost, reducing the S/N for continuous analyzers. Single Ion Monitoring (SIM) enhances the S/N (signal/noise) by setting the mass spectrometer at the m/z for an ion of interest. Since the instrument is not scanned the S/N improves, but any information about other ions is lost. Pulsed mass analyzers are the other major class of mass analyzer. These are less common but they have some distinct advantages. These instruments collect an entire mass spectrum from a single pulse of ions. This results in a signal to noise advantage similar to Fourier transform or multichannel spectroscopic techniques. Pulsed analyzers include time-of-flight, ion cyclotron resonance, and quadrupole ion trap mass spectrometers.

Magnetic Sector Analyzer

Magnetic Sector. The first mass spectrometer, built by J.J. Thompson in 1897, used a magnet to measure the m/z value of an electron. Magnetic sector instruments have higher resolution and greater mass range than quadrupole instruments, but they require larger vacuum pumps and often scan more slowly. The typical mass range is to m/z 5000, but this may be extended to m/z 30,000. Magnetic sector instruments are often used in series with an electric sector, described below, for high resolution and tandem mass spectrometry experimentation. Magnetic sector instruments separate ions in a magnetic field according to the momentum and charge of the ion. Ions are accelerated from the source region into the magnetic sector by

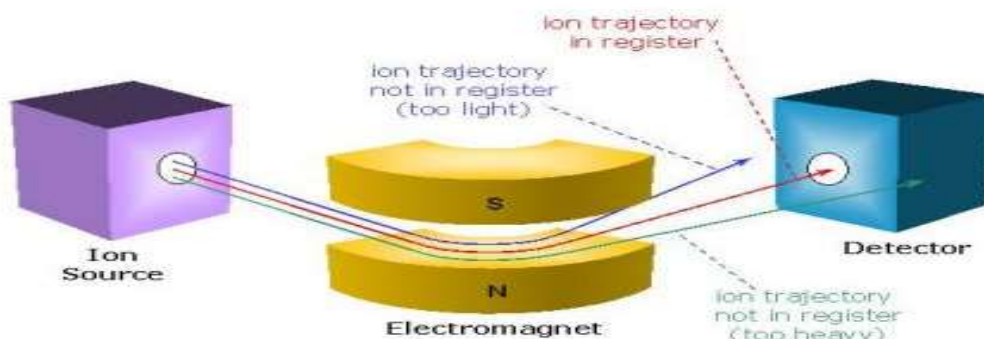
BMS COLLEGE OF ENGINEERING, BANGALORE-19
DEPARTMENT OF BIOTECHNOLOGY
Faculty : Dr. V. Saisha

a 1 to 10 kV electric field. This acceleration is significantly greater than the 100 V acceleration typical for a quadrupole instrument. Since the ions are charged, as they move through the magnetic sector, the magnetic field bends the ion beam in an arc. The radius of this arc (r) depends upon the momentum of the ion, the charge of the ion (C) and the magnetic field strength (B).

Ions with greater momentum will follow an arc with a larger radius. This separates ions according to their momentum, so magnetic sectors are often called momentum analyzers. The momentum of the ion is the product of the mass (m) and the velocity (v). The charge of the ion is

the product of the charge number of the ion (z) and the charge of an electron (e). The velocity of an ion is determined by the acceleration voltage in the source region (V) and the mass to charge ratio (m/z) of the ion. Only one m/z value will satisfy for a given radius, magnetic field and acceleration voltage. Other m/z ions will travel a different radius in the magnetic sector.

Older magnetic sector instruments use a photographic plate to simultaneously detect ions at different radii. Since each m/z has a different radius, they strike the photographic plate at a different location. Modern instruments have a set of slits at a fixed radius to transmit a single m/z to the detector. The mass spectrum is scanned by changing the magnetic field or the acceleration voltage to transmit different m/z ions. Some new instruments use multichannel diode array detectors to simultaneously detect ions over a range of m/z values.



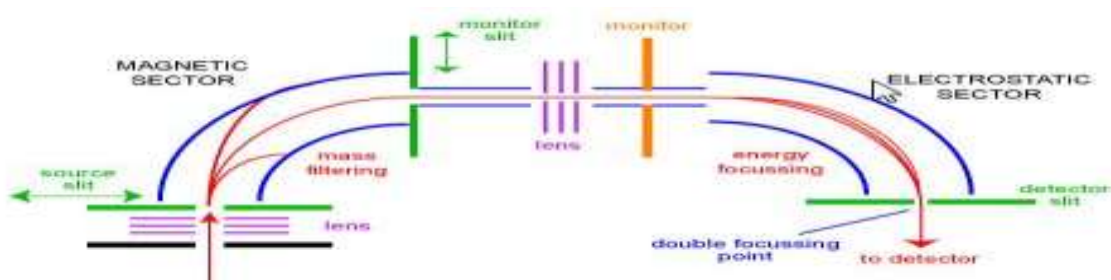
Electric sector analyzer

An electric sector consists of two concentric curved plates. A voltage is applied across these plates to bend the ion beam as it travels through the analyzer. The voltage is set so that the beam follows the curve of the analyzer. The radius of the ion trajectory (r) depends upon the kinetic energy of the ion (V) and the potential field (E) applied across the plates. An electric sector will not separate ions accelerated to a uniform kinetic energy. The radius of the ion beam is independent of the ion's mass to charge ratio so the electric sector is not useful as a standalone mass analyzer. An electric sector is, however, useful in series with a magnetic sector. The mass resolution of a magnetic sector is limited by the kinetic energy distribution (ΔV) of the ion beam. This kinetic energy distribution results from variations in the acceleration of ions produced at different locations in the source and from the initial kinetic energy distribution of

BMS COLLEGE OF ENGINEERING, BANGALORE-19
DEPARTMENT OF BIOTECHNOLOGY
Faculty : Dr. V. Saisha

the molecules. An electric sector significantly improves the resolution of the magnetic sector by reducing the kinetic energy distribution of the ions.

The effect of the electric sector is shown in Figure below.



The quadrupole mass spectrometer

The quadrupole mass spectrometer is the most common mass analyzer.

Its compact size, fast scan rate, high transmission efficiency and modest vacuum requirements are ideal for small inexpensive instruments. Most quadrupole instruments are limited to unit m/z

resolution and have a mass range of m/z 1000.. In the mass spectrometer, an electric field accelerates ions out of the source region and into the quadrupole analyzer. The analyzer consists of four rods or electrodes arranged across from each other. As the ions travel through the quadrupole they are filtered according to their m/z value so that only a single m/z value ion can strike the detector. The m/z value transmitted by the quadrupole is determined by the Radio Frequency (RF) and Direct Current (DC) voltages applied to the electrodes. These voltages produce an oscillating electric field that functions as a bandpass filter to transmit the selected m/z value.

The RF voltage rejects or transmits ions according to their m/z value by alternately focusing them in different planes. The four electrodes are connected in pairs and the RF potential is applied between these two pairs of electrodes. During the first part of the RF cycle the top and bottom rods are at a positive potential and the left and right rods are at a negative potential. This squeezes positive ions into the horizontal plane. During the second half of the RF cycle the polarity of the rods is reversed. This changes the electric field and focuses the ions in the vertical plane. The quadrupole field continues to alternate as the ions travel through the mass analyzer. This causes the ions to undergo a complex set of motions that produces a three-dimensional wave.

The quadrupole field transmits selected ions because the amplitude of this three dimensional wave depends upon the m/z value of the ion, the potentials applied, and the RF frequency. By selecting an appropriate RF frequency and potential, the quadrupole acts like a high pass filter, transmitting high m/z ions and rejecting low m/z ions. The low m/z ions have a greater acceleration rate so the wave for these ions has a greater amplitude. If this amplitude is

BMS COLLEGE OF ENGINEERING, BANGALORE-19
DEPARTMENT OF BIOTECHNOLOGY
Faculty : Dr. V. Saisha

great enough the ions will collide with the electrodes and can not reach the detector. The low m/z

value cutoff of the quadrupole is changed by adjusting the RF potential or the RF frequency. Any ions with a m/z greater than this cutoff are transmitted by the quadrupole.

A DC voltage is also applied across the rods of the analyzer. This potential combined with the RF potential acts like a low pass filter to reject high m/z ions. Because they respond quickly to the changing RF field the motion of the low m/z ions is dominated by the RF potential.

The combination of high and low pass filters produced by the RF and DC potentials is adjusted to only transmit the selected m/z value. All ions above or below the set m/z value are rejected by the quadrupole filter. The RF and DC fields are scanned (either by potential or frequency) to collect a complete mass spectrum. Quadrupole mass analyzers are often called mass filters because of the similarity between m/z selection by a quadrupole and wavelength selection by an optical filter or frequency selection by an electronic filter.

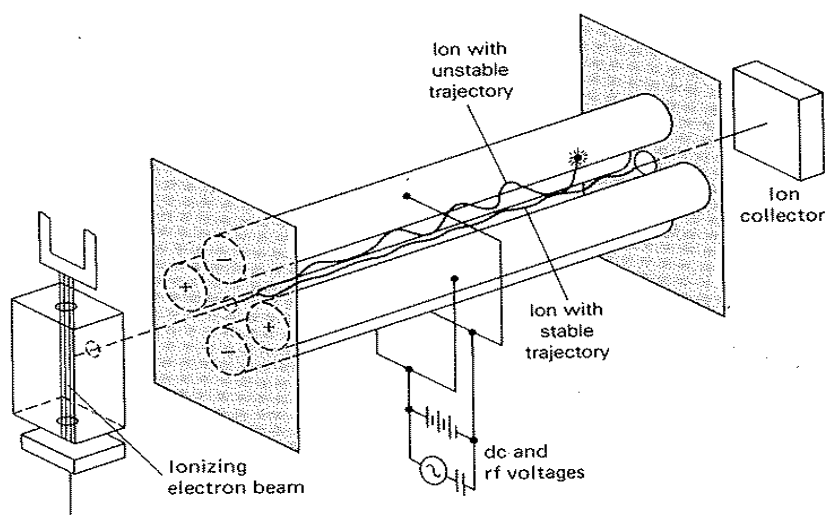
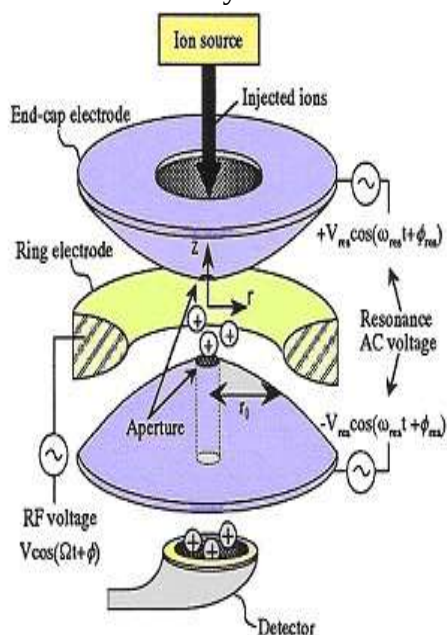


FIGURE 18-7 A quadrupole mass spectrometer. (From D. Lichtman, *Res. Dev.*, **1964**, 15(2), 52. With permission, © 1964 Cahners Publishing Company.)

Ion Trap Mass Analyzer

BMS COLLEGE OF ENGINEERING, BANGALORE-19
DEPARTMENT OF BIOTECHNOLOGY
Faculty : Dr. V. Saisha

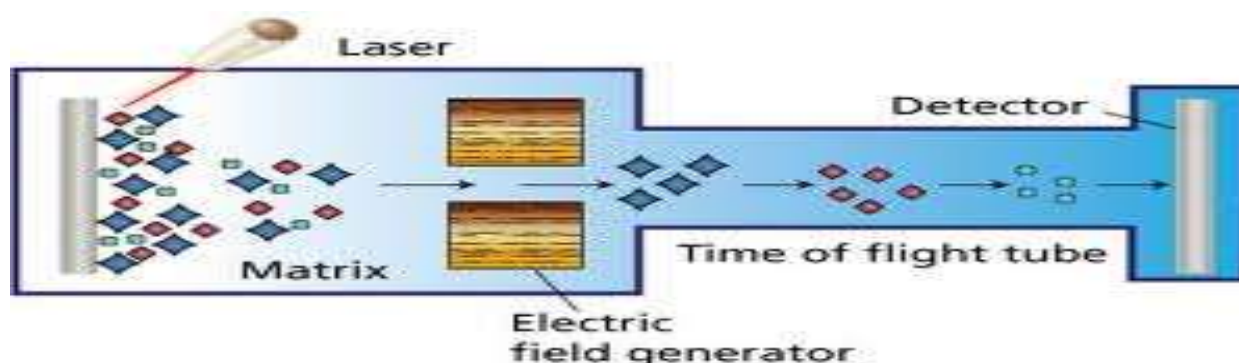
Ion traps are ion trapping devices that make use of a three-dimensional quadrupole field to trap and mass-analyze ions. Invented by Wolfgang Paul (Nobel Prize 1989)



Time-of-Flight. The time-of-flight (TOF) mass analyzer separates ions in time as they travel down a flight tube. This is a very simple mass spectrometer that uses fixed voltages and does not require a magnetic field. These instruments have high transmission efficiency, no upper m/z limit, very low detection limits, and fast scan rates.

In the source of a TOF analyzer, a packet of ions is formed by a very fast (ns) ionization pulse. These ions are accelerated into the flight tube by an electric field (typically 2-25 kV) applied between the backing plate and the acceleration grid. Since all the ions are accelerated across the same distance by the same force, they have the same kinetic energy. Because velocity (v) is dependent upon the kinetic energy (E) and mass (m), lighter ions will travel faster. E is determined by the acceleration voltage of the instrument (V) and the charge of the ion (e). After the ions accelerate, they enter a 1 to 2 meter flight tube. The ions drift through this field-free region at the velocity reached during acceleration. At the end of the flight tube, they strike a

detector. The time delay (t) from the formation of the ions to the time they reach the detector depends upon the length of the drift region (L), the mass-to-charge ratio of the ion, and the acceleration voltage in the source. Low m/z ions will reach the detector first. The mass spectrum is obtained by measuring the detector signal as a function of time for each pulse of ions produced in the source region. Because all the ions are detected, TOF instruments have very high transmission efficiency, which increases the S/N level.



Quadrupole Ion Trap. The Quadrupole ion storage trap mass spectrometer (QUISTOR) is a recently developed mass analyzer with some special capabilities.

QUISTORs are very sensitive, relatively inexpensive, and scan fast enough for GC/MS experiments. The sensitivity of the QUISTOR results from trapping and then analyzing all the ions produced in the source. Since all the ions are detected, the S/N is high.

The QUISTOR consists of a doughnut shaped ring electrode and two endcap electrodes.

A combination of RF and DC voltages is applied to the electrodes to create a quadrupole electric field similar to the electric field for the quadrupole mass analyzer. This electric field traps ions in a potential energy well at the center of the analyzer. The mass spectrum is acquired by scanning the RF and DC fields to destabilize low mass to charge ions. These destabilized ions are ejected through a hole in one end cap electrode and strike a detector. The mass spectrum is generated by scanning the fields so that ions of increasing m/z value are ejected from the cell and detected.

Ion Cyclotron Resonance The Ion Cyclotron Resonance (ICR) mass spectrometer uses a superconducting magnet to trap ions in a small sample cell. This type of mass analyzer has extremely high mass resolution and is also useful for tandem mass spectrometry experiments. These instruments are very expensive and are typically used for specialized research applications. The ICR traps ions in a magnetic field that causes ions travel in a circular path. This is similar to the path of an ion in a magnetic sector, but the ions are not traveling as fast and the magnetic field is stronger. As a result the ions are contained in the small volume of the trap.

The ion's cyclotron frequency is the angular frequency of an ion's orbit. This frequency is determined by the magnetic field strength (B) and the m/z value of the ion.

After ions are trapped in this cell they are detected by measuring the signal at this cyclotron frequency. This signal is measured by placing electrodes on each side of the ions circular orbit. An RF voltage is applied to the transmitter electrodes at the cyclotron frequency of the ion of interest. This RF energy moves ions at the applied frequency to a larger orbit. As these ions travel around the ICR cell they are close enough to the receiver electrodes to induce a capacitive current. This capacitive current oscillates at the cyclotron frequency and is detected as the signal.

The ICR is also used as a Fourier Transform Mass Spectrometer (FT-MS). Instead of

BMS COLLEGE OF ENGINEERING, BANGALORE-19
DEPARTMENT OF BIOTECHNOLOGY
Faculty : Dr. V. Saisha

using a single excitation frequency, a fast RF pulse is applied to the transmitter electrodes. This simultaneously excites all the ions and produces a signal at the cyclotron frequency of each m/z

ion present. A complete mass spectrum is obtained by using the Fourier transform to convert this signal from the time domain to the frequency domain.

Detectors:

Detection of ions is based upon their charge or momentum. For large signals a faraday cup is used to collect ions and measure the current. Older instruments used photographic plates to measure the ion abundance at each mass to charge ratio.

detectors commonly used include: electron multipliers, channeltrons and multichannel plates.

. A detector is selected for its speed, dynamic range, gain, and geometry. Some detectors are sensitive enough to detect single ions.

Faraday Cup

A Faraday cup is a metal (conductive) cup designed to catch charged particles in vacuum. The resulting current can be measured and used to determine the number of ions or electrons hitting the cup. The Faraday cup is named after Michael Faraday. When a beam or packet of ions hits the metal it gains a small net charge while the ions are neutralized. The metal can then be discharged to measure a small current equivalent to the number of impinging ions. Essentially the faraday cup is part of a circuit where ions are the charge carriers in vacuum and the faraday cup is the interface to the solid metal where electrons act as the charge carriers (as in most circuits). By measuring the electrical current (the number of electrons flowing through the circuit per second) in the metal part of the circuit the number of charges being carried by the ions in the vacuum part of the circuit can be determined. For a continuous beam of ions (each with a single charge)

- $N/t = I/e$, where N is the number of ions observed in a time t (in seconds), I is the measured current (in amperes) and e is the elementary charge (about 1.60×10^{-19} C).

Electron multiplier

electron multiplier is a vacuum-tube structure that multiplies incident charges. resulting in secondary emission. If an electric potential is applied between this metal plate and yet another, the emitted electrons will accelerate to the next metal plate and induce secondary emission of still more electrons

Array detectors : Detectors arranged in an array or matrix. Uses microchannel plates as ion to electron convertors. Can be electro-optical. Photons produced strike a photosensitive plate and the current produced is recorded.

Vacuum system

All mass spectrometers operate at very low pressure (high vacuum). This reduces the chance of ions colliding with other molecules in the mass analyzer. Any collision can cause the ions to react, neutralize, scatter, or fragment. All these processes will interfere with the mass spectrum. To minimize collisions, experiments are conducted under high vacuum conditions,

BMS COLLEGE OF ENGINEERING, BANGALORE-19
DEPARTMENT OF BIOTECHNOLOGY
Faculty : Dr. V. Saisha

typically 10⁻² to 10⁻⁵ Pa (10⁻⁴ to 10⁻⁷ torr) depending upon the geometry of the instrument. This high vacuum requires two pumping stages. The first stage is a mechanical pump that provides

rough vacuum down to 0.1 Pa (10⁻³ torr). The second stage uses diffusion pumps or turbomolecular pumps to provide high vacuum. ICR instruments have even higher vacuum requirements and often include a cryogenic pump for a third pumping stage. The pumping system is an important part of any mass spectrometer but a detailed discussion is beyond the scope of this paper.

Data system:

The final component of a mass spectrometer is the data system. This part of the instrument has undergone revolutionary changes in the past twenty years. It has evolved from photographic plates and strip chart recorders to data systems that control the instrument, acquire

hundreds of spectra in a minute and search tens of thousands of reference spectra to identify an unknown.

Interpretation

Although mass spectrometry is a very sensitive instrumental technique, there are other techniques with picogram detection limits. In addition to sensitivity, however, mass spectrometry also is also useful for identifying the chemical structure of this picogram sample. Since the mass spectrum is a fingerprint of the molecular structure, comparison to a computer databases can be used to identify an unknown compound. This is often done using Probability Based Matching (PBM), a popular pattern recognition technique. Although these computer searches are convenient and powerful, it is important to understand how to interpret a mass spectrum. A computer only compares the unknown spectrum to the library spectra and offers a selection of compounds in the database that produce similar spectra. This computer search is very useful and it makes interpretation much easier, but there are limits to the computer search.

Different Types of MS

- GC-MS - Gas Chromatography MS
 - separates volatile compounds in gas column and ID's by mass
- LC-MS - Liquid Chromatography MS
 - separates delicate compounds in HPLC column and ID's by mass
- MS-MS - Tandem Mass Spectrometry
 - separates compound fragments by magnetic field and ID's by mass
- LC/LC-MS/MS-Tandem LC and Tandem MS
 - Separates by HPLC, ID's by mass and AA sequence

BMS COLLEGE OF ENGINEERING, BANGALORE-19
DEPARTMENT OF BIOTECHNOLOGY
Faculty : Dr. V. Saisha

Applications of mass spectrometry

Biotechnology applications

- Automated high throughput analysis
- Screening of biological samples
- Pharmacokinetics
- LC-MS – separation and identification of components of complex mixtures – Normally LC-ESI, now increasingly LC-Maldi-ToF
- Intact virus analysis
- Cell imaging and tissue Imaging (Maldi)
- Protein and peptide analysis for MW determination
- Protein Identification and profiling using digests and data base searching
- Protein post-translational modification
- Protein structure characterisation
- Maldi-Imaging
- Oligo-nucleotide analysis – Confirmation of purity of synthetic oligo's
- Carbohydrate analysis

MALDI-TOF MS

Matrix-assisted laser desorption/ionization time of flight mass spectrometry

Involves Ionization of molecules via laser pulses and separates molecules according to mass to charge ratio. Mainly used for detection of large biomolecules

Development of MALDI-TOF

Developed in 1988 by Professor Franz Hillenkamp and a group of his assistants at Germany University of Munster

Designed to enhance mass-spec. by solving two main problems : Thermal instability and low volatility and analysis of large and heavy biomolecules

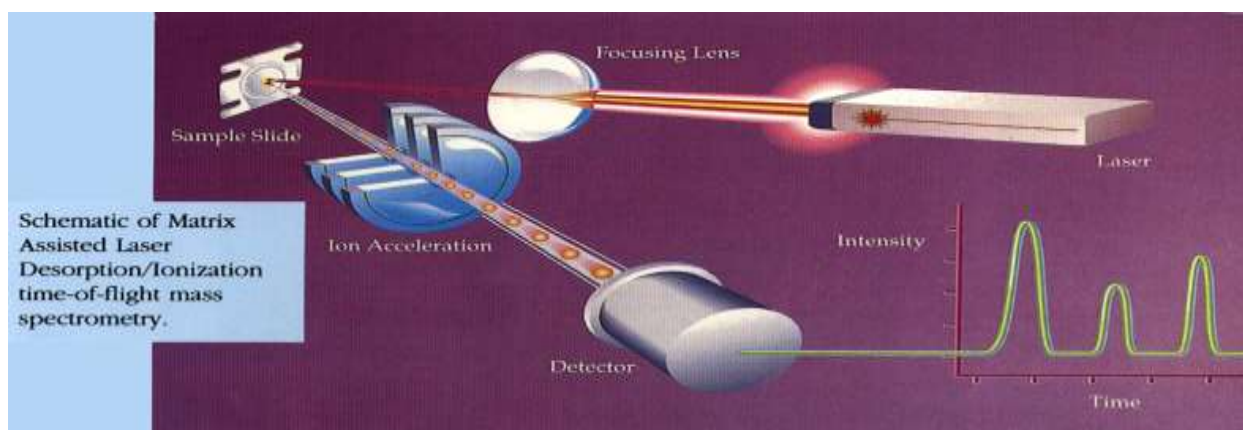
The Principles of MALDI

First, the polymer needs to be dissolved in a solvent. Eg. Proteins are usually dissolved in water, sometimes acetonitrile is added. Next, after the compound is in solution, a matrix needs to be added. Different matrices work better with different compounds. However, all matrices need absorb UV radiation. Some common matrices are trans-cinnamic acid or 2,5-dihydroxybenzoic acid. Matrix is generally added in an amount that is greater than 10^4 times the sample, ensuring that the matrix absorbs a majority of the radiation rather than the sample. This will prevent unwanted sample fragmentation. Another important aspect of the matrix is that it serves to isolate polymers from one another. Finally, the matrix serves as a source of protons for the sample to ionize. After the sample has been prepared, the solution is loaded into

BMS COLLEGE OF ENGINEERING, BANGALORE-19
DEPARTMENT OF BIOTECHNOLOGY
Faculty : Dr. V. Saisha

the sample chamber, which is then vacuum pumped to evacuate all of the air in the chamber. At this time, the solvent evaporates, leaving the sample in a dispersed compound containing both the matrix and the sample.

At this point, the laser shoots short pulses of light, in the 330-360 nm range, at the sample, causing it to essentially explode. The matrix is vaporized, and the sample is ionized into the +1 or -1 state. Usually, the polymers are too big and heavy to evaporate, but at these high temperatures and low pressures, evaporation or desorption occurs. Thus, we are left with ionized polymers in the gaseous phase.



Factors Affecting Quality of Output

- Evaporation rate
- Sample concentration
- Baseline calculations
- Scanning settings
- Size of crystals
- Energy of laser
- Detector voltage
- Accelerator voltage
- Extraction time
- Number of laser shots

Uses of MALDI-TOF

- Used to characterize and identify large molecules
- Used in pharmaceutical for QC, monitoring of enzyme reactions
- Used in DNA sequencing for forensics
- Used to identify different strains of viruses to help develop vaccines

Advantages / Disadvantages

Advantages

1. A very small sample size is needed. As little as 1 pmol can give a very resolved spectra.

BMS COLLEGE OF ENGINEERING, BANGALORE-19
DEPARTMENT OF BIOTECHNOLOGY
Faculty : Dr. V. Saisha

2. It is often difficult to get large biomolecules/polymers into the gas phase to use in mass spec. Using the low temperatures and high pressure, it is possible to get large molecules into the gas phase with fragmenting them.

3. Unlike SEC (Size Exclusion Chromatography), samples aren't compared to any other standards, they are absolute measurements of mass.

Disadvantages

1. It is often difficult to find suitable matrices to isolate specific polymers. The matrix not only has to isolate the molecule, but also must absorb large amounts of UV radiation so that the sample is not fragmented, but instead vaporized.
2. Being that this is a "soft" ionization technique, a majority of the energy is used volatilizing the matrix rather than exciting the ions. Thus, it is possible for ions to relax from the excited state after some collisions.
3. Due to the extreme sensitivity, contaminants can easily interfere with the produced spectra. For example, trace amounts of buffer salts will produce such intense peaks that they will effectively drain out the peaks of the sample. Thus, when these contaminants can be removed, the sample should be prepared at a very high concentration.

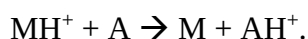
Features of MALDI-TOF MS

- Soft ionization - analyze intact biomolecules and synthetic polymers
- Fast data acquisition
- Easy to use and maintain,
- High sensitivity, superior mass resolution and accuracy

Protocol :

MALDI: Matrix Assisted Laser Desorption Ionization

1. Sample (A) is mixed with excess matrix (M) and dried on a MALDI plate.
2. Laser flash ionizes matrix molecules.
3. Sample molecules are ionized by proton transfer from matrix:



Time-of-flight mass analyzer

- Ions are formed in pulses.
- Small ions reach the detector before large ones.

BMS COLLEGE OF ENGINEERING, BANGALORE-19
DEPARTMENT OF BIOTECHNOLOGY
Faculty : Dr. V. Saisha

- Measures the time for ions to reach the detector.

Calibration of the mass scale The mass-to-charge ratio of an ion is proportional to the square of its drift time given as

$$\frac{m}{z} = \frac{2r^2K}{L^2}$$

t = Drift time

L = Drift length

m = Mass

K = Kinetic energy of ion

z = Number of charges on ion

MALDI TOF Hardware

Laser, Attenuator and Prism

Nitrogen laser at 337 nm, 3 ns wide pulses, 20 Hz. Laser attenuator varies the intensity of the laser hitting the sample. Prism deflects the laser beam into the ion source.

Sample Plate and Sample Stage

An accelerating voltage is applied to the sample plate in the range 15-25 kV.

Variable Voltage Grid

A grid 1-2 mm above the sample plate with an additional voltage to fine-tune ion acceleration

Ground Grid

Grounded surface defines end of acceleration region

Grounded Aperture

Entrance to flight tube

Vacuum System

High vacuum is required to avoid ion collisions

Flight tube

A field free region where ions drift at a velocity inversely proportional to the square root of their mass/charge.

Linear Detector

Measures the ion abundance in linear mode (no reflector used) and sends a signal to the digitizer.

Reflector

A single stage gridded ion mirror that subjects the ions to a uniform repulsive electric field to reflect them. It is tilted by 1° in the DE-STR to focus the ions on to the detector

Collision Cell

Gas cell for collision induced dissociation (CID) to enhance fragmentation in PSD analysis

Reflector Detector

Measures ions reflected by the mirror. In the DE-STR this is a 6-10 mm pore size micro-channel plate.

Timed Ion Selector

A velocity selector that allows a single precursor ion of a selected mass and their fragment ions

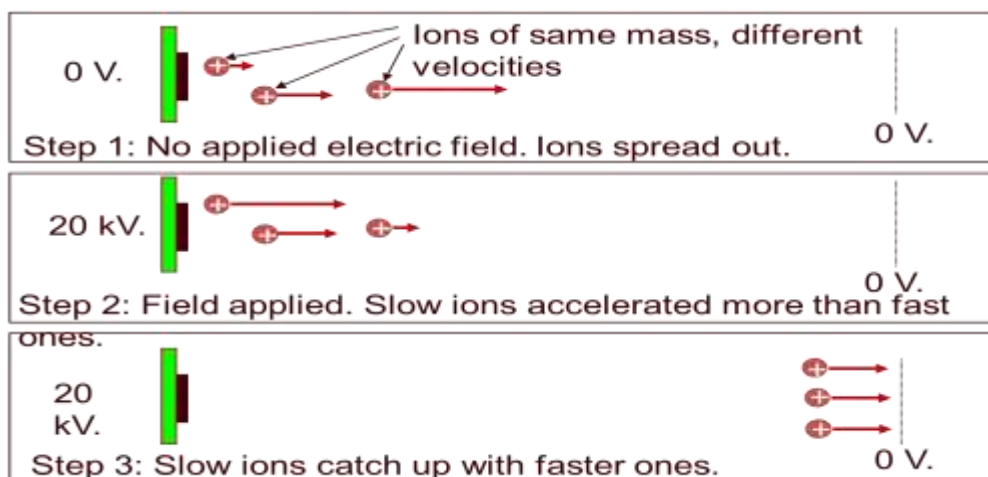
BMS COLLEGE OF ENGINEERING, BANGALORE-19
DEPARTMENT OF BIOTECHNOLOGY
Faculty : Dr. V. Saisha

to pass to the detector. A Bradbury-Neilson gate is used.

The problem: Peaks are inherently broad in MALDI-TOF spectra (poor mass resolution).

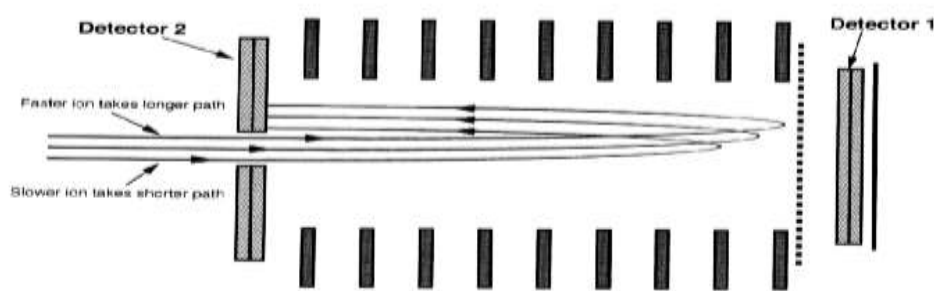
The cause: Ions of the same mass coming from the target have different speeds. This is due to uneven energy distribution when the ions are formed by the laser pulse.

Delayed Extraction (DE) improves performance



What is a reflector TOF analyzer?

A single stage gridded ion mirror that subjects the ions to a uniform repulsive electric field to reflect them. The reflector or ion mirror compensates for the initial energy spread of ions of the same mass coming from the ion source, and improves resolution.



A

reflector focuses ions to give better mass resolution

LC MS

Need for LCMS

MS analysis of components of complex samples may be complicated by selective desorption of sample components. By first fractionating samples by HPLC and following this with MS analysis, we can combine high-resolution chromatographic separation with high-resolution analysis (i.e. mass determination). This is called HPLC/MS or LC/MS. MS can be regarded as essentially a detector for the LC system, but one which gives useful structural information about each component of the sample.

Essential requirement

need for an interface between the two systems. The ion spray interface is particularly useful for

BMS COLLEGE OF ENGINEERING, BANGALORE-19
DEPARTMENT OF BIOTECHNOLOGY
Faculty : Dr. V. Saisha

protein and peptide samples .it facilitates the high flow-rates of LC systems

Techniques for adaptation of LC and MS

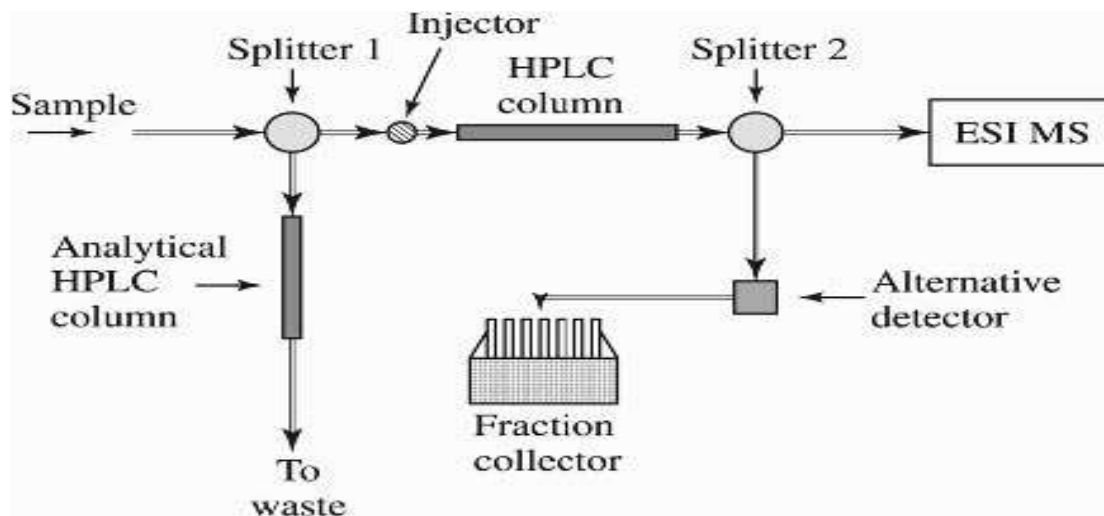
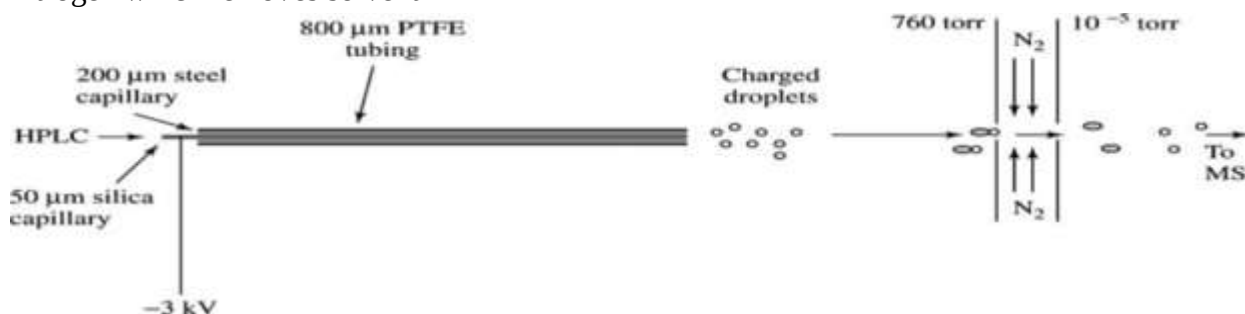
enlargement of MS pumping capacity

solvent (usually volatile organic solvents) could be removed prior to introduction of sample components into the high vacuum

region of the MS by an analyte-enrichment interface

Post column splitting and diversion of solvent flow

The ionspray interface.: Sample flows through HPLC and is converted into a fine mist of electrically-charged droplets by electrospray). These droplets are rapidly dried by a flow of nitrogen which removes solvent



Experimental setup featuring splitting of solvent flow and use of small-diameter HPLC.

Analytical HPLC of sample is facilitated by splitter 1 while splitter 2 allows collection of sample components.

Common ionization modes

ESI and FAB

MALDI

Atmospheric Pressure Chemical Ionisation

Analyzers

Quadrupole Analysers

BMS COLLEGE OF ENGINEERING, BANGALORE-19
DEPARTMENT OF BIOTECHNOLOGY
Faculty : Dr. V. Saisha

Time-of-flight Analysers

Liquid Chromatography Considerations

Flow Rate

While standard ESI sources can generally handle flow rates up to 1 mL/min, lower flow rates in the range 0.05 to 0.2 mL/min result in improved sensitivity. Columns with 1.0 or 2.1 mm diameters are therefore well suited to direct coupling with these ion sources. Higher sensitivities and resolutions can be obtained with capillary columns and sub μ L/min flow rates.

Mobile Phase: Typical solvents used in reverse and normal phase LC (e.g. water, acetonitrile, methanol, ethanol, chloroform) are compatible with ESI. It should be noted that a grade of solvent (including water) that is suitable for a conventional LC separation may not always be suitable for an LC-MS based separation. The use of buffers containing inorganic ions such as phosphate and sodium acetate should be avoided. They cause significant ion suppression, can create MS adducts of sodium and potassium, and can quickly contaminate the ion source. Higher organic content of the mobile phase can result in improvements in ionisation efficiency in ESI, and this may affect the choice of separation mode.

Resolution and Through-put Baseline separation of peaks is generally required in conventional LC with UV detection due to the non-specific nature of the detector, especially when wavelengths below 250 nm are used. In contrast, if the peaks have independent MS signals, complete chromatographic separation is not required in LC-MS. Therefore, lower resolution, shorter columns are often used in LC-MS assays with consequently shorter run times, although ion suppression effects may be a limitation. Guard columns can sometimes provide sufficient separation from interferences. Chromatographic resolution also impacts on the scanning speed of the mass spectrometer. To achieve accurate integration it is desirable to have at least 10 scans across the chromatographic peak.

Quantitation It is generally difficult to perform quantitative determinations using absolute MS responses. Internal standards are usually required to achieve reliable and accurate quantitative results. -stable isotope dilution

Method of Optimization

The actual conditions are highly dependent on the nature of the analyte and the LC separation, making it difficult to give generic conditions. Each analyte requires individual optimization. The nature of the analyte will largely determine the optimal ionization method and polarity used.

Applications

Biochemical Screening for Genetic Disorders

Therapeutic Drug Monitoring and Toxicology

Vitamins and Related Metabolites responses

Advantages

superior specificity and sensitivity compared to direct injection methods

sensitive, quantitative, multiplexed assays of plasma peptides and proteins

potential replacements for existing immunoassays.

capacity to multiplex several analytes within a single analytical run with minimal incremental cost

BMS COLLEGE OF ENGINEERING, BANGALORE-19
DEPARTMENT OF BIOTECHNOLOGY
Faculty : Dr. V. Saisha

BMS COLLEGE OF ENGINEERING, BANGALORE-19
DEPARTMENT OF BIOTECHNOLOGY
Faculty : Dr. V. Saisha

VOLTAGE AND PATCH CLAMP TECHNIQUES

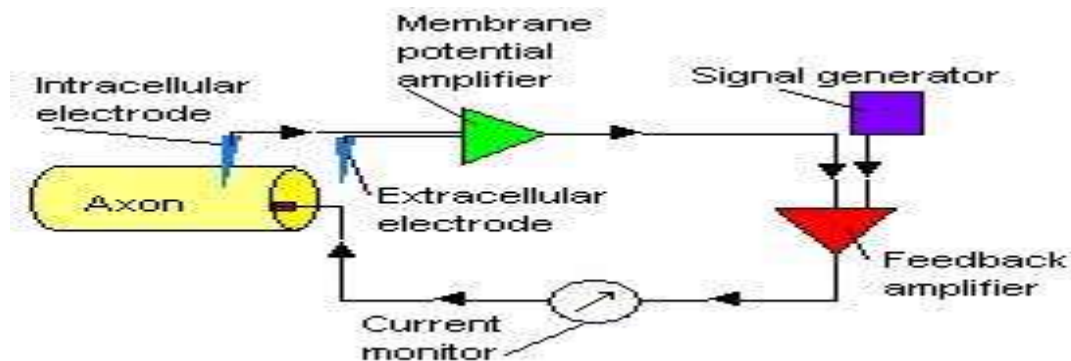
Techniques in electrophysiology which are used to study the electrical properties of biological cells and tissues. It involves the measurement of voltage change or change in current.

Voltage clamp technique

Technique to measure the ion currents through the membrane of excitable cells, such as neurons, while holding the membrane voltage at a set level. Cell membranes of excitable cells contain many different kinds of ion channels, some of which are voltage gated. The voltage clamp allows the membrane voltage to be manipulated independently of the ionic currents, allowing the current-voltage relationships of membrane channels to be studied.

The concept of the voltage clamp is attributed to Kenneth Cole and George Marmont in the 1940s. Cole discovered that it was possible to use two electrodes and a feedback circuit to keep the cell's membrane potential at a level set by the experimenter.

Technique:



The voltage clamp is a current generator with two electrodes. Transmembrane voltage is recorded through a "voltage electrode", relative to ground, and a "current electrode" passes current into the cell. The experimenter sets a "holding voltage", or "command potential", and the voltage clamp uses negative feedback to maintain the cell at this voltage. The electrodes are connected to an amplifier, which measures membrane potential and feeds the signal into a feedback amplifier. This amplifier also gets an input from the signal generator that determines the command potential, and it subtracts the membrane potential from the command potential ($V_{\text{command}} - V_m$), magnifies any difference, and sends an output to the current electrode. Whenever the cell deviates from the holding voltage, the operational amplifier generates an "error signal", that is the difference between the command potential and the actual voltage of the cell. The feedback circuit passes current into the cell to reduce the error signal to zero.

BMS COLLEGE OF ENGINEERING, BANGALORE-19
DEPARTMENT OF BIOTECHNOLOGY
Faculty : Dr. V. Saisha

Thus, the clamp circuit produces a current equal and opposite to the ionic current. This can be measured, giving an accurate reproduction of the currents flowing across the membrane.

Applications of voltage clamp amplifier

Voltage clamp amplifier is used for measuring voltage current relationship between the ion channels or voltage gated ion channels.

These are applicable to excited cells like cardiomyocytes, pancreatic cells(beta cells of pancreas), neurons, muscle fibre, ion channels of bacteria.

Variations of the voltage clamp technique

Two-electrode voltage clamp using microelectrodes

this works on the same principle as standard voltage clamps, but in contrast, the two electrodes used in this technique are glass pipettes with very fine tips (smaller than 1 micrometer), allowing for clamping of cells smaller in size.. These electrodes are filled with a conductive solution, for example, 3M KCl.

Disadvantages

1.The microelectrodes used are poorer conductors, and sometimes cannot pass current rapidly enough to compensate for cellular current.

2.space clamp. Microelectrodes can provide only a spatial point source of current that might not uniformly affect different parts of an irregularly shaped cell.

Dual-cell voltage clamp

The dual-cell voltage clamp technique is a specialized variation of the two electrode voltage clamp, and is only used in the study of gap junction channels. Gap junctions are pores which directly link two cells through which ions and small metabolites flow freely. When two cells in which gap junction proteins are expressed either endogenously or via injection of mRNA, a junction channel will form between the cells. Commonly, the Xenopus laevis oocyte expression system is used. Since two cells are present in the system, two sets of electrodes are used. A recording electrode and a current injecting electrode are inserted into each cell, and each cell is clamped individually (each set of electrodes is attached to a separate apparatus, and integration of data is performed by computer). To record junctional conductance, the current is varied in the first cell while the recording electrode in the second cell records any changes in V_m for the second cell only. (The process can be reversed with the stimulus occurring in the second cell and recording occurring in the first cell.) Since no variation in current is being induced by the electrode in the recorded cell, any change in voltage must be induced by current crossing into the recorded cell, through the gap junction channels, from the cell in which the current was varied.

BMS COLLEGE OF ENGINEERING, BANGALORE-19
DEPARTMENT OF BIOTECHNOLOGY
Faculty : Dr. V. Saisha

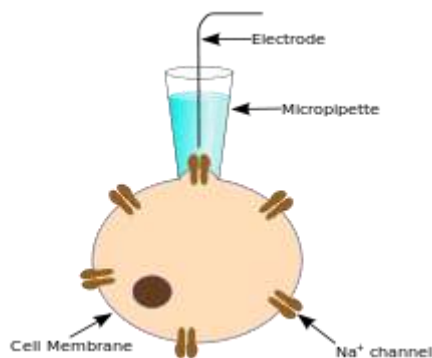
Single-electrode voltage clamp

This category describes a set of techniques in which one electrode is inserted into the cell. This single electrode carries out the functions of both current injection and recording.

PATCH CLAMP TECHNIQUE

Patch clamp technique was developed by Sakmann and Neher in 1970. Patch clamp technique is modification of basic voltage clamp. Patch clamp recording reveals the transitions between two conductance states of an ion

Allows the study of single or multiple ion channels in cells. The technique can be applied to a wide variety of cells, but is especially useful in the study of excitable cells such as neurons, cardiomyocytes, muscle fibers and pancreatic beta cells. It can also be applied to the study of bacterial ion channels in specially prepared giant spheroplasts.



Patch clamp recording uses, Uses a glass micropipette as an electrode that has an open tip diameter of about one micrometer, a size enclosing a membrane surface area or "patch" that often contains just one or a few ion channel molecules.. The pipette is filled with a solution dependent on the specific study or patch clamp technique being used.i.e a solution matching the ionic composition of the bath solution, as in the case of cell-attached recording, or the cytoplasm for whole-cell recording. A metal electrode (chlorided silver wire) in contact with this solution conducts electrical changes to a voltage clamp amplifier The micropipette is pressed against a cell membrane and suction is applied to assist in the formation of a high resistance seal between the glass and the cell membrane (a "gigaohm seal" or "gigaseal," since the electrical resistance of that seal is in excess of a gigaohm). The high resistance of this seal makes it possible to electronically isolate the currents measured across the membrane patch with little competing noise, as well as providing some mechanical stability to the recording. The investigator can change the composition of this solution or add drugs to study the ion channels under different conditions. In patch clamping a single electrode is used to patch clamp the cell membrane enabling the voltage to be maintained at a constant level while changes in current are recorded. Patch clamp recording uses a single electrode to record currents. Many patch clamp amplifiers do not use true voltage clamp circuitry but instead are differential amplifiers

BMS COLLEGE OF ENGINEERING, BANGALORE-19
DEPARTMENT OF BIOTECHNOLOGY
Faculty : Dr. V. Saisha

that use the bath electrode to set the zero current level. This allows a researcher to keep the voltage constant while observing changes in current. Alternatively, the cell can be current clamped in whole-cell mode, keeping current constant while observing changes in membrane voltage.

Variations

There are 4 major types of variations in patch clamp technique. They are:

- Whole cell patch clamp
- Cell attached patch clamp
- Inside out patch clamp
- Outside out patch clamp

.Whole-cell recording or whole-cell patch: Whole-cell patch and perforated patch allow the researcher to study the electrical behavior of the entire cell, instead of single channel currents. It involves recording currents through multiple channels at once, over the membrane of the entire cell. The electrode is left in place on the cell, but more suction is applied to rupture the membrane patch, thus providing access to the intracellular space of the cell. The advantage of whole-cell patch clamp recording over sharp microelectrode recording is that the larger opening at the tip of the patch clamp electrode provides lower resistance and thus better electrical access to the inside of the cell. A disadvantage of this technique is that the volume of the electrode is larger than the cell, so the soluble contents of the cell's interior will slowly be replaced by the contents of the electrode. This is referred to as the electrode "dialyzing" the cell's contents. Thus, any properties of the cell that depend on soluble intracellular contents will be altered. The pipette solution used usually approximates the high-potassium environment of the interior of the cell. Generally speaking, there is a period at the beginning of a whole-cell recording, lasting approximately 10 minutes, when one can take measurements before the cell has been dialyzed. The whole-cell patch, which allows for low resistance electrical access to the inside of a cell, has now largely replaced high resistance microelectrode recording techniques to record currents across the entire cell membrane

Perforated patch : In this variation of whole-cell recording, the experimenter forms the gigaohm seal, but does not use suction to rupture the patch membrane. Instead, the electrode solution contains small amounts of an antifungal or antibiotic agent, such as amphotericin-B, nystatin, or gramicidin. As the antibiotic molecules diffuse into the membrane patch, they form small perforations in the membrane, providing electrical access to the cell interior. This has the advantage of reducing the dialysis of the cell that occurs in whole-cell recordings, but also has several disadvantages. First, the access resistance is higher, relative to whole-cell, due to the partial membrane occupying the tip of the electrode (access resistance being the sum of the electrode resistance and the resistance at the electrode-cell junction). This will decrease electrical access and thus decrease current resolution, increase recording noise, and magnify any series resistance error. Second, it can take a significant amount of time for the antibiotic to perforate the membrane (10–30 minutes, though this can be reduced with properly shaped electrodes). Third, the membrane under the electrode tip is weakened by the perforations

BMS COLLEGE OF ENGINEERING, BANGALORE-19
DEPARTMENT OF BIOTECHNOLOGY
Faculty : Dr. V. Saisha

formed by the antibiotic and can rupture. If the patch ruptures, the recording is then in whole-cell mode, with antibiotic contaminating the inside of the cell.

Cell-attached or on-cell patch: Cell-attached patch techniques are used to study the behavior of individual ion channels in the section of membrane attached to the electrode. The electrode is sealed to the patch of membrane, and the cell remains intact. This allows for the recording of currents through single ion channels in that patch of membrane, without disrupting the interior of the cell. For ligand-gated ion channels or channels that are modulated by metabotropic receptors, the neurotransmitter or drug being studied is usually included in the pipette solution, where it can contact what had been the external surface of the membrane. While the resulting channel activity can be attributed to the drug being used, it is usually not possible to then change the drug concentration. The technique is thus limited to one point in a dose response curve per patch. Usually, the dose response is accomplished using several cells and patches. However, voltage-gated ion channels can be clamped at different membrane potentials using the same patch. This results in graded channel activation, and a complete I-V (current-voltage) curve can be established with only one patch.

Inside-out patch: The inside-out and outside-out techniques are called "excised patch" techniques, because the patch is excised (removed) from the main body of the cell. These patch techniques are also used to study the behavior of individual ion channels in the section of membrane attached to the electrode. After the gigaseal is formed, the micropipette is quickly withdrawn from the cell, thus ripping the patch of membrane off the cell, leaving the patch of membrane attached to the micropipette, and exposing the intracellular surface of the membrane to the external media. This is useful when an experimenter wishes to manipulate the environment at the intracellular surface of ion channels. For example, channels that are activated by intracellular ligands can then be studied through a range of ligand concentrations.

Outside-out patch : After the whole-cell patch is formed, the electrode can be slowly withdrawn from the cell, allowing a bulb of membrane to bleb out from the cell. When the electrode is pulled far enough away, this bleb will detach from the cell and reform as a convex membrane on the end of the electrode (like a ball open at the electrode tip), with the original outside of the membrane facing outward from the electrode. Single channel recordings are possible in this conformation if the bleb of membrane is small enough. Outside-out patching gives the experimenter the opportunity to examine the properties of an ion channel when it is isolated from the cell, and exposed to different solutions on the extracellular surface of the membrane. The experimenter can perfuse the same patch with different solutions, and if the channel is activated from the extracellular face, a dose-response curve can then be obtained. This is the distinct advantage of the outside-out patch relative to the cell-attached method. However, it is more difficult to accomplish, as more steps are involved in the patching process.

Loose patch : Loose patch clamp is different in that it employs a loose seal rather than the tight gigaseal used in the conventional technique. A significant advantage of the loose seal is that

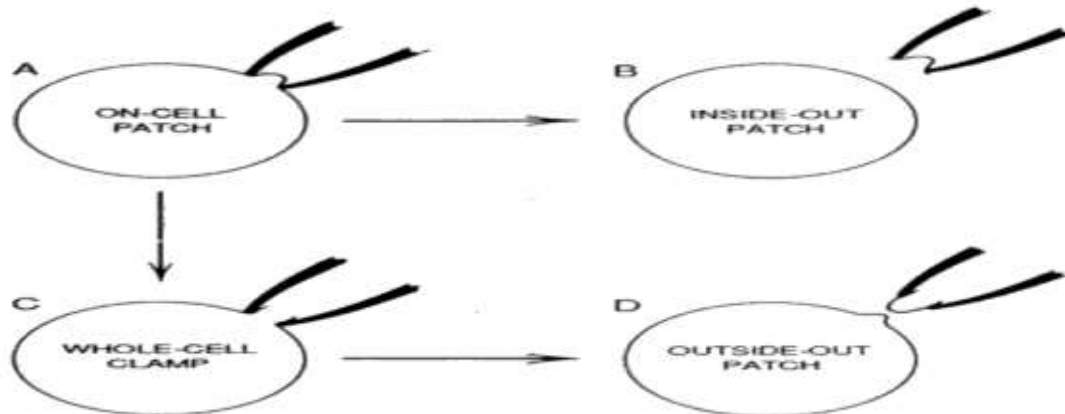
BMS COLLEGE OF ENGINEERING, BANGALORE-19
DEPARTMENT OF BIOTECHNOLOGY
Faculty : Dr. V. Saisha

the pipette that is used can be repeatedly removed from the membrane after recording, and the membrane will remain intact. This allows for repeated measurements in a variety of locations on the same cell without destroying the integrity of the membrane. A major disadvantage is that the resistance between the pipette and the membrane is greatly reduced, allowing current to leak through the seal. This leakage can be corrected for, however, which offers the opportunity to compare and contrast recordings made from different areas on the cell of interest.

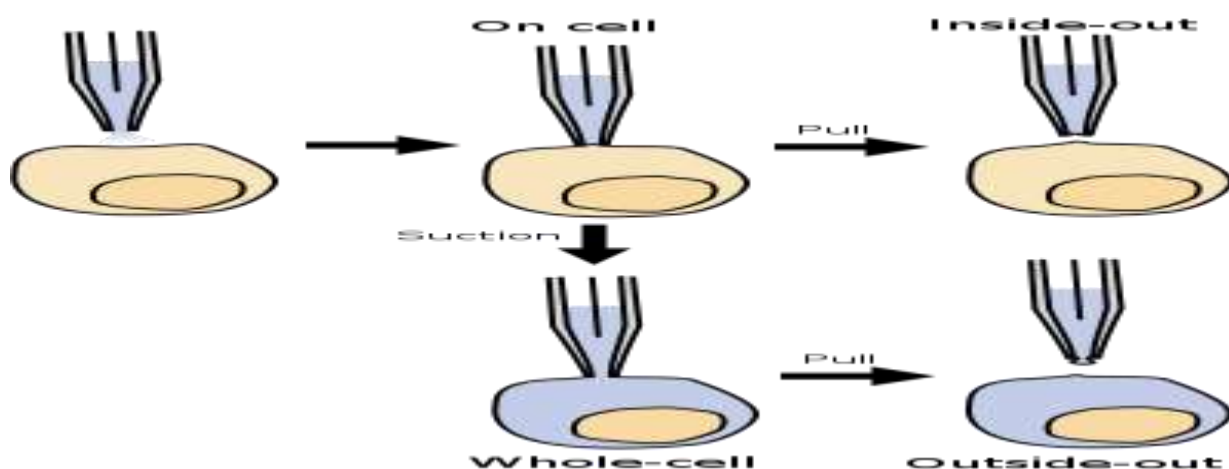
Automatic patch clamping: Automated patch clamp systems have recently been developed, in order to inexpensively collect large amounts of data in a shorter period of time. Such systems typically include a single-use microfluidic device, either an injection molded or a PDMS cast chip, to capture a cell or cells, and an integrated electrode.

Applications of patch clamp technique :

The technique is used to study excitable cells ie, those that produce a small electrical current when stimulated. Most cell types of clinical matter concerning biophysical investigation became accessible with it. Numerous inherited diseases were due to a faulty channel function (ion channel mutation)



BMS COLLEGE OF ENGINEERING, BANGALORE-19
DEPARTMENT OF BIOTECHNOLOGY
Faculty : Dr. V. Saisha



References :

1. Physical Biochemistry-Principles and applications by -David Sheehan ., Wiley publications.
2. Biological Spectroscopy by J. D. Campbell and R. A.Dwek, Plenum Press.
3. Biophysical Chemistry by Cantor R., and Schimmel P.R.,Freeman
4. Principles and Techniques of Practical Biochemistry –Keith Wilson ., Cambrigde Univ. Press.
5. Web Sources.