PAVENDAR BHARATHIDASAN COLLEGE OF ARTS AND SCIENCE

APPLIED BIOCHEMISTRY

II B.Sc., Biotechnology

16SACBT2

Unit 1

Basic Principles of Sedimentation

- Particles or cells in a liquid suspension sediment at the bottom of a container due to gravity.
- The time required for such separation is usually very long. The sedimentation effect is mainly influenced by the Earth's gravitational field (g=98.1cms-2).

- Centrifugation is a technique of separating substances which involves the application of centrifugal force.
- The particles are separated from a solution according to their size, shape, density, the viscosity of the medium and rotor speed.

Types of Centrifuge

- **Low-Speed Centrifuge**
- **High-Speed Centrifuges**
- **Ultracentrifuges**

Low-speed centrifugation

A separation method where the components of a sample are separated on the basis of their density in a centrifuge according to the centrifugal force they experience. Samples are spun at <5000 rpm.

1) Most laboratories have a standard low-speed centrifuge used for routine sedimentation of heavy particles

2) The low-speed centrifuge has a maximum speed of 4000-5000rpm

3) These instruments usually operate at room temperatures with no means of temperature control. 4) Two types of rotors are used in it,

- Fixed angle
- Swinging bucket.

4) It is used for sedimentation of red blood cells until the particles are tightly packed into a pellet and supernatant is separated by decantation.

High-Speed Centrifuges

1. High-speed centrifuges are used in more sophisticated biochemical applications, higher speeds and temperature control of the rotor chamber are essential.
2. The high-speed centrifuge has a maximum speed of 15,000 – 20,000 RPM
3. The operator of this instrument can carefully control speed and temperature which is required for sensitive biological samples.

Three types of rotors are available for high-speed centrifugation-

- Fixed angle
- Swinging bucket
- Vertical rotors

Ultracentrifuges

1. It is the most sophisticated instrument.
2. Ultracentrifuge has a maximum speed of 65,000 RPM (100,000's x g).
3. Intense heat is generated due to high speed thus the spinning chambers must be refrigerated and kept at a high vacuum.
4. It is used for both preparative work and analytical work.

Differential Pelleting (differential centrifugation)

- It is the most common type of centrifugation employed.
- Tissue such as the liver is homogenized at 32 degrees in a sucrose solution that contains buffer.
- The homogenate is then placed in a centrifuge and spun at constant centrifugal force at a constant temperature.
- After some time a sediment forms at the bottom of a centrifuge called pellet and an overlying solution called supernatant.
- The overlying solution is then placed in another centrifuge tube which is then rotated at higher speeds in progressing steps.

Density Gradient Centrifugation

- This type of centrifugation is mainly used to purify viruses, ribosomes, membranes, etc.
- A sucrose density gradient is created by gently overlaying lower concentrations of sucrose on higher concentrations in centrifuge tubes
- The particles of interest are placed on top of the gradient and centrifuge in ultracentrifuges.
- The particles travel through the gradient until they reach a point at which their density matches the density of surrounding sucrose.
- The fraction is removed and analyzed.

Rate-Zonal Density-Gradient Centrifugation

- Zonal centrifugation is also known as band or gradient centrifugation
- It relies on the concept of sedimentation coefficient (i.e. movement of sediment through the liquid medium)
- In this technique, a density gradient is created in a test tube with sucrose and high density at the bottom.
- The sample of protein is placed on the top of the gradient and then centrifuged.
- With centrifugation, faster-sedimenting particles in sample move ahead of slower ones i.e. sample separated as zones in the gradient.
- The protein sediment according to their sedimentation coefficient and the fractions are collected by creating a hole at the bottom of the tube.

Isopynic Centrifugation

- The sample is loaded into the tube with the gradient-forming solution (on top of or below pre-formed gradient, or mixed in with self-forming gradient)
- The solution of the biological sample and cesium salt is uniformly distributed in a centrifuge tube and rotated in an ultracentrifuge.
- Under the influence of centrifugal force, the cesium salts redistribute to form a density gradient from top to bottom.
- Particles move to point where their buoyant density equals that part of gradient and form bands. This is to say the sample molecules move to the region where their density equals the density of gradient.
- It is a "true" equilibrium procedure since depends on bouyant densities, not velocities

Eg: CsCl, NaI gradients for macromolecules and nucleotides – "self-forming" gradients under centrifugal force.

Application of Centrifugation

- To separate two miscible substances
- To analyze the hydrodynamic properties of macromolecules
- Purification of mammalian cells

- Fractionation of subcellular organelles (including membranes/membrane fractions) Fractionation of membrane vesicles
- Separating chalk powder from water
- Removing fat from milk to produce skimmed milk
- Separating particles from an air-flow using cyclonic separation
- The clarification and stabilization of wine
- Separation of urine components and blood components in forensic and research laboratories
- Aids in the separation of proteins using purification techniques such as salting out, e.g. ammonium sulfate precipitation.

Sedimentation velocity

- Sedimentation velocity (SV-AUC) is an analytical ultracentrifugation method that measures the rate at which molecules move in response to centrifugal force generated in a centrifuge.
- This sedimentation rate provides information about both the molecular mass and the shape of molecules.

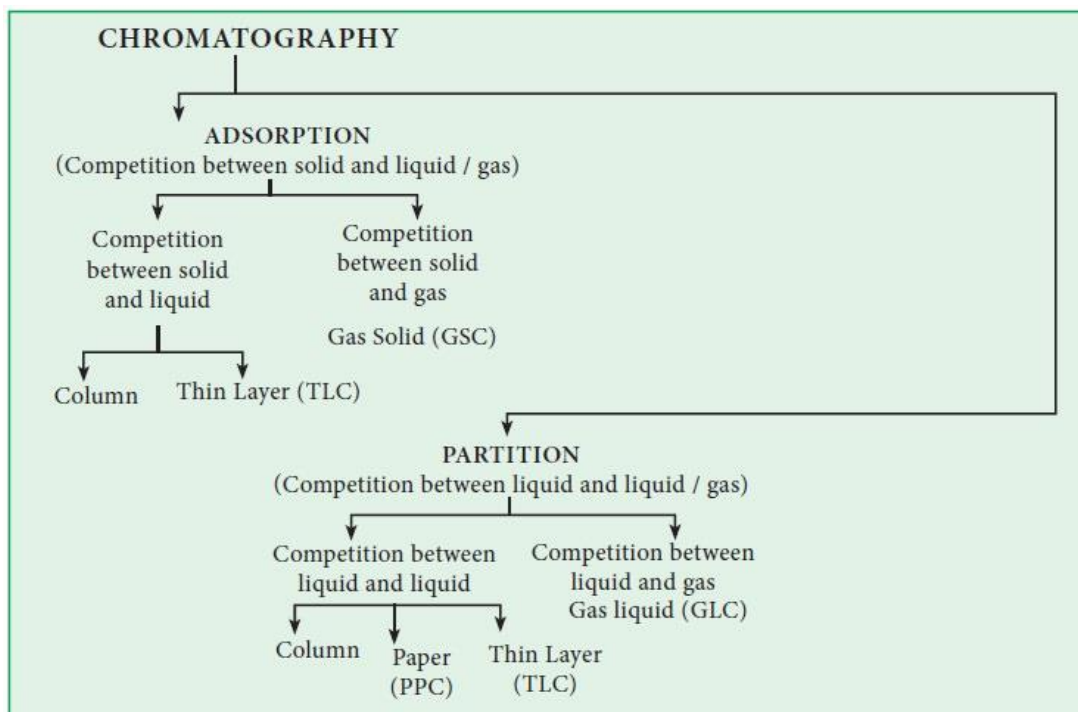
Sedimentation equilibrium

- Sedimentation equilibrium (SE-AUC) is an analytical ultracentrifugation method for measuring protein molecular masses in solution and for studying protein-protein interactions.
- It is particularly valuable for: establishing whether the native state of a protein is a monomer, dimer, trimer, etc.

Unit 2

Chromatography

- Chromatography is a laboratory technique for the separation of a mixture.
- The mixture is dissolved in a fluid called the mobile phase, which carries it through a structure holding another material called the stationary phase.
- The various constituents of the mixture travel at different speeds, causing them to separate.
- Chromatography is used in industrial processes to purify chemicals, test trace quantities of substances, separate chiral compounds and quality control test products.
- Chromatography is the physical process of separating or analyzing complex mixtures.
- The Rf value is defined as the ratio of the distance moved by the solvent (i.e. the coloring or pigment being tested) and the distance moved by the solvent (known as the Solvent front) along the paper, where both distances are measured from the common origin or application baseline.



- Chromatography is used for quality analyses and checker in the food industry, by identifying and separating, analyzing additives, vitamins, preservatives, proteins, and amino acids. Chromatography like HPLC is used in DNA fingerprinting and bioinformatics.
- Affinity chromatography is a method of separating biochemical mixture based on a highly specific interaction between antigen and antibody, enzyme and substrate, receptor and ligand, or protein and nucleic acid.
- High Performance Liquid Chromatography (HPLC) is a form of column chromatography that pumps a sample mixture or analyte in a solvent (known as the mobile phase) at high pressure through a column with chromatographic packing material (stationary phase).

Technique	Stationary phase	Mobile phase	Format	Principal sorption mechanism
Paper chromatography (PC)	Paper (Cellulose)	Liquid	Planar	Partition (adsorption, ion-exchange, exclusion)
Thin-layer chromatography (TLC)	Silica, cellulose, ion-exchange resin, controlled porosity solid	Liquid	Planar	Adsorption (partition, ion-exchange, exclusion)
Gas chromatography (GC)				
Gas-liquid chromatography (GLC)	Liquid	Gas	Column	Partition
Gas-solid chromatography (GSC)	Solid	Gas	Column	Adsorption
Liquid chromatography (LC)				
High-performance liquid chromatography (HPLC)	Solid or bonded-phase	Liquid	Column	Modified partition (adsorption)
Size-exclusion chromatography (SEC)	Controlled porosity solid	Liquid	Column	Exclusion
Ion-exchange chromatography (IEC) Ion chromatography (IC)	Ion-exchange resin or bonded-phase	Liquid	Column	Ion-exchange
Chiral chromatography (CC)	Solid chiral selector	Liquid	Column	Selective adsorption

Type of Chromatography	Applications in the Real World	Why and What is it
Liquid Chromatography	testing water samples to look for pollution	Used to analyze metal ions and organic compounds in solutions. It uses liquids which may incorporate hydrophilic, insoluble molecules.
Gas Chromatography	detecting bombs in airports, identifying and quantifying such drugs as alcohol, being used in forensics to compare fibres found on a victim	Used to analyze volatile gases. Helium is used to move the gaseous mixture through a column of absorbent material.
Thin-Layer Chromatography	detecting pesticide or insecticide residues in food, also used in forensics to analyze the dye composition of fibres	Uses an absorbent material on flat glass plates. This is a simple and rapid method to check the purity of the organic compound.
Paper Chromatography	separating amino acids and anions, RNA fingerprinting, separating and testing histamines, antibiotics	The most common type of chromatography. The paper is the stationary phase. This uses capillary action to pull the solutes up through the paper and separate the solutes.

GC-MS, LC-MS

- Both methods separate the chemicals by chromatography first, then further examine and identify them by the mass spectrometer.
- The only difference is that LC-MS uses a solvent as its mobile phase, while GC-MS uses inert gases (like helium) in the same capacity.
- After digestion, LC-MS is used for peptide mass fingerprinting, or LC-MS/MS (tandem MS) is used to derive the sequences of individual peptides.
- LC-MS/MS is most commonly used for proteomic analysis of complex samples where peptide masses may overlap even with a high-resolution mass spectrometry.

Table 1: Comparison of GC–MS and LC–MS for trace analysis of drugs in biological fluids		
Aspect	GC–EI-MS	LC–ESI-MS/MS
General application	Volatile, thermally stable molecules (low- to mid-polarity); low-volatility compounds need to be derivatized	Nonvolatile, polar and ionic molecules (mid- to high-polarity)
Cost	~$100k	~$200k
Available? Established?	Yes, yes	Yes, yes
Sample preparation for biological fluids	Liquid–liquid extraction; solid-phase extraction; derivatization (to make polar compounds more volatile); finish in volatile solvent	Dilute-and-inject (urine); Protein precipitation; liquid–liquid extraction; solid-phase extraction; finish in polar solvent (water ideal)
Limits of detection and quantification	Nanogram (10^{-9} g) to picogram (10^{-12} g)	Picogram to femtogram (10^{-15} g)
Specificity?	Yes, via EI mass spectrum (library matching; abundant diagnostic fragment ions from EI); lack of molecular ion is a problem	Yes, via MS/MS; monitor specific fragments of desired molecular ion using triple quadrupole; no universal library matching
Matrix effects?	Minimal	Yes, require stable isotopically labelled internal standards for each analyte

Unit 3

Electrophoresis

- Electrophoresis is a general term that describes the migration and separation of charged particles (ions) under the influence of an electric field.
- An electrophoretic system consists of two electrodes of opposite charge (anode, cathode), connected by a conducting medium called an electrolyte.

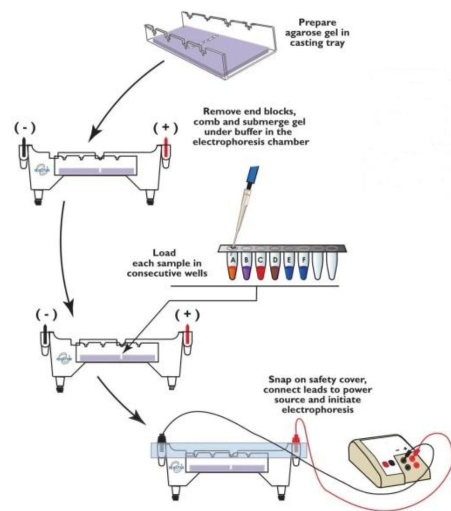
Electrophoresis Principle and its types

- Charged macromolecules are placed in the electric field move towards the negative or positive pole based on their charge.
- This technique is divided into two types viz slab electrophoresis and capillary electrophoresis.

- Electrophoresis equipment applies an electric charge to molecules, causing them to migrate towards their oppositely charged electrode.
- The technique is found in all research and clinical laboratories utilizing DNA and protein applications, and is divided into gel and capillary techniques.
- Gel electrophoresis is a technique used to separate DNA fragments (or other macromolecules, such as RNA and proteins) based on their size and charge.
- Electrophoresis involves running a current through a gel containing the molecules of interest.
- Electrophoresis has a wide application in separating and analyzing biomolecules such as proteins, plasmids, RNA, DNA, nucleic acids.

Agarose gel electrophoresis

- Agarose gel electrophoresis is a method of gel electrophoresis used in biochemistry, molecular biology, genetics, and clinical chemistry to separate a mixed population of macromolecules such as DNA or proteins in a matrix of agarose, one of the two main components of agar.
- Agarose is a polysaccharide, generally extracted from certain red seaweed.
- It is a linear polymer made up of the repeating unit of agarobiose, which is a disaccharide made up of D-galactose and 3,6-anhydro-L-galactopyranose.

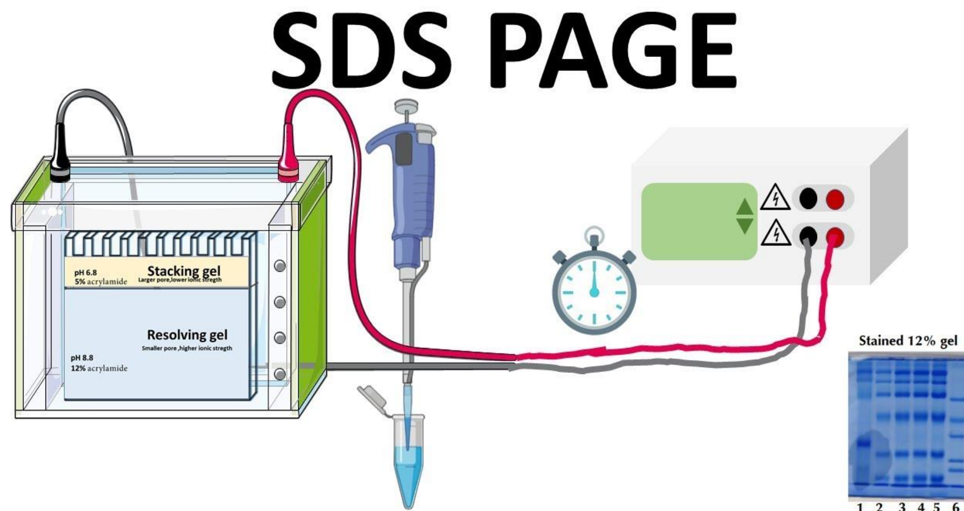


Application

- The agarose gel electrophoresis is widely employed in molecular genetics, especially in PCR and PCR related techniques such as DNA fingerprinting, RFLP, AFLP, and RAPD analysis.
- It allows the purification of DNA fragments and separation of DNA fragments for sequencing and other downstream application.

SDS-PAGE

- It uses sodium dodecyl sulfate (SDS) molecules to help identify and isolate protein molecules.
- SDS-PAGE is a discontinuous electrophoretic system developed by Ulrich K. Laemmli which is commonly used as a method to separate proteins with molecular masses between 5 and 250 KDa.
- PAGE (Polyacrylamide Gel Electrophoresis), is an analytical method used to separate components of a protein mixture based on their size.
- The technique is based upon the principle that a charged molecule will migrate in an electric field towards an electrode with opposite sign.



Application of SDS-PAGE

- It is used to measure the molecular weight of the molecules. It is used to estimate the size of the protein. It is used to estimate the purity of the proteins.
- It is used in Western Blotting and protein ubiquitination.

Isoelectric Focusing.

- Isoelectric focusing (IEF) is steady-state electrophoresis in a pH gradient.
- Ionic compounds migrate to the point in the gradient where they have zero overall charge and therefore zero net mobility.
- For proteins this means that they migrate to their isoelectric point (PI value).

Two-dimensional gel electrophoresis

- Two-dimensional gel electrophoresis or 2D-PAGE is the primary technique for proteomics work.
- It separates the complex mixture of samples using two different properties of the proteins.
- In the first dimension, proteins are separated by the pI value and in the second dimension by the relative molecular weight.

Unit 4

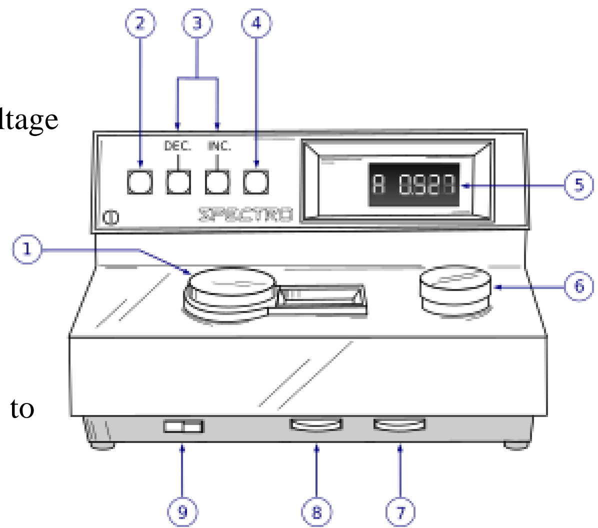
Spectroscopy

- Spectroscopy in Biology and Chemistry discusses the use of thermal neutron diffraction and inelastic scattering, and the related techniques of x-ray diffraction, Raman and Rayleigh scattering, in investigating biological macromolecules and chemical systems.
- Beer's Law may be written simply as: $A = \varepsilon bc$. where A is absorbance (no units) ε is the molar absorptivity with units of L mol-1 cm-1 (formerly called the extinction coefficient) b is the path length of the sample, usually expressed in cm.
- Transmission of light is the moving of electromagnetic waves (whether visible light, radio waves, ultraviolet, etc.) through a material.
- This transmission can be reduced, or stopped, when light is reflected off the surface or absorbed by the molecules in the material.
- Photoelectric colorimeter is the instruments, with the help of which quantitative analysis of colored solutions is possible.

- The photoelectric colorimeters are of two types.
- The unnecessary radiations are absorbed and we get light of only one colour.
- UV/Vis spectroscopy is routinely used in analytical chemistry for the quantitative determination of different analytes, such as transition metal ions, highly conjugated organic compounds, and biological macromolecules.
- Spectroscopic analysis is commonly carried out in solutions but solids and gases may also be studied.

A colorimeter is a device used in colorimetry that measures the absorbance of particular wavelengths of light by a specific solution. It is commonly used to determine the concentration of a known solute in a given solution by the application of the Beer–Lambert law, which states that the concentration of a solute is proportional to the absorbance.

The essential parts of a colorimeter are:



(1) Wavelength selection, (2) Printer button, (3) Concentration factor adjustment, (4) UV mode selector (Deuterium lamp), (5) Readout, (6) Sample compartment, (7) Zero control (100% T), (8) Sensitivity switch, (9)ON/OFF switch

- a **light source** (often an ordinary low-voltage filament lamp)
- an adjustable aperture
- a set of colored filters
- a cuvette to hold the working solution
- a detector (usually a photoresistor) to measure the transmitted light
- a meter to display the output from the detector

In addition, there may be:

- a voltage regulator, to protect the instrument from fluctuations in mains voltage;
- a second light path, cuvette and detector. This enables comparison between the working solution and a "blank", consisting of pure solvent, to improve accuracy.

A spectrophotometer is an instrument containing a monochromator, a device which produces a light beam containing wavelengths in a narrow band around a selected wavelength, and a means of measuring the ratio of that beam's intensity as it enters and leaves a cuvette99This describes a single-beam photometer.

	Colorimeter (Filter Photometer)	Spectrophotometer
Light Source	LED, Fixed Wavelength	Lamp (Tungsten, Xenon, Deuterium), Wavelength Range
Wavelength Selector	Color Filter, Fixed Wavelength	Monochromator, Wavelength Range
Portability	Stationary parts, light weight, good for field use	Moving parts, heavier, good for bench use. The DR 1900 can be used in the field. It is lighter and battery operated, but still has some moving parts.
Parameters	Single or Limited Number of Parameters determined by Fixed Wavelengths	Numerous Parameters determined by the Wavelength Range.

Nuclear magnetic resonance

- Nuclear magnetic resonance spectroscopy is widely used to determine the structure of organic molecules in solution and study molecular physics, crystals as well as non-crystalline materials.
- NMR is also routinely used in advanced medical imaging techniques, such as in magnetic resonance imaging (MRI).

- Electron Spin Resonance (ESR), often called Electron Paramagnetic Resonance (EPR), is similar to Nuclear Magnetic Resonance (NMR), the fundamental difference being that ESR is concerned with the magnetically induced splitting of electronic spin states, while NMR describes the splitting of nuclear spin states.
- Simplified **Principle** of **Electron Spin Resonance** (**ESR**) With an **ESR** instrument, a static magnetic field and microwaves are used to observe the behavior of the unpaired electrons in the material being studied.
- **ESR** is used to observe and measure the absorption of microwave energy by unpaired electrons in a magnetic field

Applications

NMR is extensively used in medicine in the form of magnetic resonance imaging. NMR is used industrially mainly for routine analysis of chemicals. The technique is also used, for example, to measure the ratio between water and fat in foods, monitor the flow of corrosive fluids in pipes, or to study molecular structures such as catalysts.

Medicine

The application of nuclear magnetic resonance best known to the general public is magnetic resonance imaging for medical diagnosis and magnetic resonance microscopy in research settings. However, it is also widely used in biochemical studies, notably in NMR spectroscopy such as proton NMR, carbon-13 NMR, deuterium NMR and phosphorus-31 NMR. Biochemical information can also be obtained from living tissue (e.g. human brain tumors) with the technique known as in vivo magnetic resonance spectroscopy or chemical shift NMR microscopy.

These spectroscopic studies are possible because nuclei are surrounded by orbiting electrons, which are charged particles that generate small, local magnetic fields that add to or subtract from the external magnetic field, and so will partially shield the nuclei. The amount of shielding depends on the exact local environment. For example, a hydrogen bonded to an oxygen will be shielded differently from a hydrogen bonded to a carbon atom. In addition, two hydrogen nuclei can interact via a process known as spin-spin coupling, if they are on the same molecule, which will split the lines of the spectra in a recognizable way.

As one of the two major spectroscopic techniques used in metabolomics, NMR is used to generate metabolic fingerprints from biological fluids to obtain information about disease states or toxic insults.

Application of ESR

- EPR/ESR spectroscopy is used in various branches of science, such as biology, chemistry and physics, for the detection and identification of free radicals in the solid, liquid, or gaseous state, and in paramagnetic centers such as F-centers. EPR is a sensitive, specific method for studying both radicals formed in chemical reactions and the reactions themselves
- Electron paramagnetic resonance (EPR) has proven itself as a useful tool in homogeneous catalysis research for characterization of paramagnetic complexes and reactive intermediates. EPR spectroscopy is a particularly useful tool to investigate their electronic structures, which is fundamental to understand their reactivity.
- Medical and biological applications of EPR also exist. Although radicals are very reactive, and so do not normally occur in high concentrations in biology, special reagents have been developed to spin-label molecules of interest. These reagents are particularly useful in biological systems. Specially-designed nonreactive radical molecules can attach to specific sites in a biological cell, and EPR spectra can then give information on the environment of these so-called spin labels or spin probes. Spin-labeled fatty acids have been extensively used to study dynamic organisation of lipids in biological membranes, lipid-protein interactions and temperature of transition of gel to liquid crystalline phases.
- EPR can be used to measure microviscosity and micropolarity within drug delivery systems as well as the characterization of colloidal drug carriers
- EPR/ESR spectroscopy has been used to measure properties of crude oil, in particular asphaltene and vanadium content. EPR measurement of asphaltene content is a function of spin density and solvent polarity.
- In the field of quantum computing, pulsed EPR is used to control the state of electron spin qubits in materials such as diamond, silicon and gallium arsenide.

Unit 5

Crystallography

- Crystallography, branch of science that deals with discerning the arrangement and bonding of atoms in crystalline solids and with the geometric structure of crystal lattices.
- Classically, the optical properties of crystals were of value in mineralogy and chemistry for the identification of substances.

- Some of the specific areas that can now be probed with X-ray crystallography include measuring the thickness of films, identifying specific crystal phases and orientations that can help to determine the catalytic activity of materials, determining the purity of a sample.

Crystal structure

- In crystallography, crystal structure is a description of the ordered arrangement of atoms, ions or molecules in a crystalline material.
- The smallest group of particles in the material that constitutes this repeating pattern is the unit cell of the structure.

  The size of the unit cell and the arrangement of atoms in a crystal may be determined from measurements of the diffraction of X-rays by the crystal, termed X-ray crystallography.

X-ray crystallography

- X-ray crystallography is used to examine a sample that is in the crystalline state.
- Crystals of many proteins and other biomoleculars have been obtained and analyzed in the X-ray beam.
- A few macromolecular crystals are discussed in the chapter through various figures.

Application of X-ray

- Projectional radiography is the practice of producing two-dimensional images using x-ray radiation. Bones contain much calcium, which due to its relatively high atomic number absorbs x-rays efficiently. This reduces the amount of X-rays reaching the detector in the shadow of the bones, making them clearly visible on the radiograph.
- The lungs and trapped gas also show up clearly because of lower absorption compared to tissue, while differences between tissue types are harder to see.

Fluoroscopy

- Fluoroscopy is an imaging technique commonly used by physicians or radiation therapists to obtain real-time moving images of the internal structures of a patient through the use of a fluoroscope. In its simplest form, a fluoroscope consists of an X-ray source and a fluorescent screen, between which a patient is placed. However, modern fluoroscopes couple the screen to an X-ray image intensifier and CCD video camera allowing the images to be recorded and played on a monitor. This method may use a contrast material.
- Examples include cardiac catheterization (to examine for coronary artery blockages) and barium swallow (to examine for esophageal disorders and swallowing disorders).

Radiotherapy

- The use of X-rays as a treatment is known as radiation therapy and is largely used for the management (including palliation) of cancer; it requires higher radiation doses than those received for imaging alone.
- X-rays beams are used for treating skin cancers using lower energy x-ray beams while higher energy beams are used for treating cancers within the body such as brain, lung, prostate, and breast.

Powder method

- The powder method is used to determine the value of the lattice parameters accurately.
- Lattice parameters are the magnitudes of the unit vectors a, b and c which define the unit cell for the crystal.
- If a monochromatic x-ray beam is directed at a single crystal, then only one or two diffracted beams may result.
- Powder diffraction is a scientific technique using X-ray, neutron, or electron diffraction on powder or microcrystalline samples for structural characterization of materials.
- An instrument dedicated to performing such powder measurements is called a powder diffractometer.

Advantages

Although it is possible to solve crystal structures from powder X-ray data alone, its single crystal analogue is a far more powerful technique for structure determination. This is directly related to the fact that information is lost by the collapse of the 3D space onto a 1D axis. Nevertheless, powder X-ray diffraction is a powerful and useful technique in its own right. It is mostly used to characterize and identify phases, and to refine details of an already known structure, rather than solving unknown structures.

Advantages of the technique are:

- Simplicity Of Sample Preparation
- Rapidity Of Measurement
- The Ability To Analyze Mixed Phases, E.G. Soil Samples
- "In Situ" Structure Determination

By contrast growth and mounting of large single crystals is notoriously difficult. In fact there are many materials for which, despite many attempts, it has not proven possible to obtain single crystals. Many materials are readily available with sufficient microcrystallinity for powder diffraction, or samples may be easily ground from larger crystals. In the field of solid-state chemistry that often aims at synthesizing new materials, single crystals thereof are typically not immediately available. Powder diffraction is therefore one of the most powerful methods to identify and characterize new materials in this field.

Particularly for neutron diffraction, which requires larger samples than X-ray diffraction due to a relatively weak scattering cross section, the ability to use large samples can be critical, although newer and more brilliant neutron sources are being built that may change this picture.

Since all possible crystal orientations are measured simultaneously, collection times can be quite short even for small and weakly scattering samples. This is not merely convenient, but can be essential for samples which are unstable either inherently or under X-ray or neutron bombardment, or for time-resolved studies. For the latter it is desirable to have a strong radiation source. The advent of synchrotron radiation and modern neutron sources has therefore done much to revitalize the powder diffraction field because it is now possible to study temperature dependent changes, reaction kinetics and so forth by means of time-resolved powder diffraction.